SELECTIVE PEPTIDE BOND FORMATION IN CAPTIVE ENVIRONMENTS

A Thesis Submitted
in Partial Fulfilment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

by

GIRIJ PAL SINGH

to the

DEPARTMENT OF CHEMISTRY

INDIAN INSTITUTE OF TECHNOLOGY KANPUR

AUGUST, 1988

Dedicated
to
My Parents

STATEMENT

I hereby declare that the matter embodied in this thesis is the result of investigations carried out by me in the Department of Chemistry, Indian Institute of Technology, Kanpur, India, under the supervision of Professor S. Ranganathan.

In keeping with the general practice of reporting scientific observations due acknowledgements have been made wherever the work embodied is based on the findings of other investigators.

Gpsingh

GIRIJ PAL SINGH

CERTIFICATE

Certified that the work contained in this thesis, entitled, "SELECTIVE PEPTIDE BOND FORMATION IN CAPTIVE ENVIRONMENTS" has been carried out by Mr. Girij Pal Singh, under my supervision and the same has not been submitted elsewhere for a degree.

Kanpur
August 1988.

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CERTIFICATE OF COURSE WORK

This is to certify that Mr. Girij Pal Singh has satisfactorily completed all the course requirement for the Ph.D. degree programme. The courses include :

Chm. 502	Advanced Organic Chemistry
Chm. 505	Principles of Organic Chemistry
Chm. 524	Modern Physical Methods in Chemistry
Chm. 525	Principles of Physical Chemistry
Chm. 545	Principles of Inorganic Chemistry
Chm. 581	Basic Biological Chemistry
Chm. 800	General Seminar
Chm. 801	Graduate Seminar
Chm. 900	Research.

Mr. Girij Pal Singh has successfully completed his Ph.D. Qualifying Examinations in Feb., 1985. He has also successfully presented his open seminar of the work embodied in this thesis.

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PREFACE

The thesis entitled, "SELECTIVE PEPTIDE BOND FORMATION IN CAPTIVE ENVIRONMENTS" consists of six parts, namely, A. Introduction, B. Background, C. Present Work, D. Spectra, E. Experimental and F. References.

SUMMARY OF THE PRESENT WORK

The work reported in the thesis outlines endeavours directed at the formation of peptide bonds in a selective manner from coded α-amino acids taking advantage of either the intrinsic order present in the crystal lattice, or the possible arrangements in aqueous media or at a micellar interface.

The diversity that is inherent in the side chains of the 20 coded amino acids would certainly lead to preferences in alignments and the choice of proximate partners. In principle, peptide bond formation, taking advantage of such preferences, would lead to preferred peptide sequences that may well be precursors to present day enzymes. The identification of such preferences, the factors that control the manifestation of such selectivity and the role played by environments in this direction are little understood. However, it was envisaged that the order that is likely to be present in selected environments could be taken advantage of, in the demonstration of selectivity in peptide bond formation.

Most amino acids form crystals whose X-ray crystallographic data show that there is a nearly universal ordering in the sense that the head to tail arrangement is consistently observed. This arrangement represents an

order, which, if taken advantage of, could lead to peptide bond formation. More interesting are examples where a pair of amino acids, whose side chains have the property to form salts, form mixed crystals. X-Ray crystallographic studies of such mixed crystals have clearly demonstrated an intrinsic order in each case, which, if taken advantage of, could lead to selective peptide bond formation. For example, the aspartic acid - histidine mixed crystals exhibit a highly ordered arrangement consisting of alternate bilayers of aspartic acid and histidine wherein all the hydrogen bonding possibilities are taken advantage of (CHART S.1* = CHART C.1). It was envisaged that peptide bond formation in a mixed crystal could be achieved without disturbing the crystal order via co-crystallizing with a carbodiimide precursor. This concept is illustrated in CHART S.2 = CHART C.2. Since the approach described above envisages the formation of the carbodiimide in a crystal lattice, the only appropriate methodology that could be adapted would be the generation of such species by photolysis. At the outset, three such photoprecursors of dimethylcarbodiimide were envisaged. Amongst these, compound (1)** is known and has been demonstrated, on photolysis, to undergo extrusion of elements of nitrogen and sulfur, leading to dimethylcarbodiimide. In the present work it was envisaged that the diazo compound (7) and the tetraamino ethylene system (9), would be even more effective in their transformation to the reactive carbodiimide (CHART S.3 = CHART C.3).

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*In charts and tables, the notation "S" stands for "Synopsis" and "C" for the section outlining the present work (SECTION C) in the thesis.

**These numbers refer to those assigned in the present work (SECTION C and SECTION E).

The preparation of compound (1) is illustrated in CHART S.4 = CHART C.4. In present endeavours to effect the preparation of the diazo compound (7) and the dimer (9), (CHARTS S.5 = CHART C.5 and CHART S.6 = CHART C.6) the methosulfate intermediate (3) (CHART S.4 = CHART C.4) was considered as an appropriate starting material for the diazo compound (7). The reaction of (3) with tosyl hydrazide gave the expected tosyl hydrazone (4) which could not be transformed to (7) under various reaction conditions. In another attempt, compound (3), with either benzhydrazide or ethyl carbazate, gave the substituted hydrazones (5a) and (5b) which were transformed to (6), the hydrazone precursor of (7). Endeavours to effect the (6) → (7) transformation by usual methods did not succeed (CHART S.5 = CHART C.5). Amongst the many efforts directed at the preparation of dimer (9), the most promising appeared to be via double extrusion of intermediate (8) arising from possible union of the tosyl hydrazone (4) with compound (1). In the event, this could not be effected (CHART S.6 = CHART C.6).

Attempts to form mixed crystals involving several amino acid pairs and the thione (1), either in water, or in aqueous acetone, invariably led to the crystallization of (1) separately. It was then envisaged that the problems associated with forming a crystal involving three components on the one hand and that possibly arising from the lack of interaction of (1) with amino acids on the other could be reduced via attachment of an appropriate ligand, either acidic or basic in nature, to (1) thus enabling salt formation of appropriate amino acids, which, hopefully, would provide crystals incorporating both the components. Such crystals would retain the property two form the carbodiimide and the principle of selectivity could be demonstrated using such systems as illustrated in CHART S.7 = CHART C.7, with the aspartic acid - Mannich base carbodiimide precursor.

Mannich base (13) was prepared from (10) and the Mannich bases (15) and (16), from (11). In addition, compounds (15) and (16) were obtained via (12), the normal N-hydroxymethyl intermediate involved in such reactions. Although the expected salts were formed on treatment of the bases (13), (15) and (16) with aspartic acid, all endeavours to obtain suitable crystal composites did not succeed. The converse strategy endeavouring to attach acidic ligands at the 4-position of compound (1) via reaction of (11) with either the $BrCH_2CO_2Et$ or the $BrCH_2CO_2H$, gave, instead of N-alkylation the S-alkylated products (20) and (21) (CHART S.8 = CHART C.8) (CHART S.9 = CHART C.9).

Efforts outlined above and several others clearly brought out, above all, the difficulties in the formation of crystal composites. Therefore, alternate methodologies for the demonstration of selective peptide bond formation were explored.

The order that exists in crystal lattice, harbouring a pair of amino acids, could logically be extended to their concentrated aqueous solutions. In this event, the condensation of amino acids, taking advantage of this order, can be achieved using water soluble carbodiimides. Such a selective peptide bond formation in a milieu is also related to the problem of peptide bond formation at the very early stages of protein evolution.

An enzyme having an amino acid sequence $1 \rightarrow n$ could be made, either by the addition of a single unit or, in a convergent approach, by joining existing preferred sequences. The efficiency of the latter approach is significantly higher and, consequently, would be favoured by nature (CHART S.10 = CHART C.11). The preferences in peptide bond formation involving the 20 coded amino acids in aqueous medium, has been examined by two methodologies. In the experimental approach pairs of amino acids in water were allowed

to undergo peptide formation using the requisite amount of water soluble carbodiimide and the products analyzed, either by preparative TLC or HPLC, thus enabling the determining of the ratio of the peptides formed amongst the 'n' number of possibilities, when amino acids 'a' and 'b' are made to combine. In the theoretical approach, a computer based analysis of a number of established protein sequences was carried out to possibly delineate preferred polypeptide units that could have served as synthons in the convergent approach which cited in (CHART S.10 = CHART C.11 ; CHART S.11 = CHART C.12).

Equivalent amounts of glutamic acid, leucine and the specifically prepared water soluble carbodiimide, 1-cyclohexyl 3(3-dimethylaminopropyl) carbodiimide metho paratoluene sulfonate (22) in clear aqueous solution was left stirred for 2 days, the resulting dipeptide mixture N,C-protected and analysed by preparative TLC, HPLC and NMR and compared with authentic samples of all possibilities, namely, BzLeu-LeuOMe (30), BzLeu-GludiOMe (31), BzGlu(γ -OMe) - LeuOMe (32), BzGlu(γ -OMe) - GludiOMe (33), BzGlu(α -OMe) - GludiOMe (34) and BzGlu(α -OMe) - LeuOMe (CHART S.12 = CHART C.14). The results of such an analysis showed that in the reaction of unprotected glutamic acid and leucine in water, in presence of the carbodiimide (22), the product percentages, as analyzed by h.p.l.c, were BzGlu (γ -OMe)-LeuOMe (72%), BzGlu(α -OMe)-GludiOMe (15%), BzLeu-LeuOMe (8%) and 1.5% of higher peptides (TABLE S.1 = TABLE C.1), thus demonstrating that regardless of finer aspects of reaction mechanisms, a clear preference for the formation of some dipeptides over others do exist.

In vitro peptide bond formation involving glutamic acid consistently show a preference for a higher reactivity of the sterically less hindered γ -carboxyl group. In the peptide bond formation involving glutamic acid that takes

place in every cell, the more hindered α-carboxyl group is involved in the formation of the peptide bond. The fact that a similar trend is evident in the present experiment (TABLE S.1 = TABLE C.1), thus mimicing the in vivo processes perhaps constitute the most important result of this experiment. The α-selectivity and the preponderance of Glu-Leu (72%) has been rationalized on the basis of the pka value differences with respect to the -α and γ- carboxyl functions of glutamic acid leading to selective bond formation involving the α-carboxyl moiety, which, in turn, forms the activated ester with the carbodiimide (22) followed by peptide bond formation with the more basic α-amino function of leucine. The very low preference exhibited in the formation of Leu-Leu (8%) and the fact that excepting in this case, the Leu residue does not appear at the amino end of the dipeptide supports the preferential formation of the glutamic acid - carbodiimide complex envisaged in CHART S.13 = CHART C.15. Several ancilliary experiments involving, individually, glutamic acid and leucine, under similar conditions, were carried out and to confirm the conclusions described above. A similar set of experiments involving glutamic acid and glycine resulted in the preferential glycine polymerization initiated by specific activation of this residue (TABLE S.1 = TABLE C.1).

The experimental complexities associated with the possible analysis of dipeptides arising from α-amino acids in aqueous medium is maximum in the case of glutamic acid with lysine, which could, in principle, as illustrated in CHART S.14 = CHART C.16, give twelve dipeptides. In the event, the reaction of glutamic acid and lysine in clear water solution with one equivalent of water soluble carbodiimide (22), followed by N,C-protection and HPLC analysis showed only two products whose nature remains to be completely established. Regardless of this, the experiment demonstrated a high order of selectivity which is supported by the fact that the reaction of equivalent amounts

of lysine and the carbodiimide (22), followed by N,C-protection, yielded largely BzLys (ω -Bz) - Lys(ω -Bz)OMe, involving exclusive peptide bond formation involving the α -amino group.

A computer programme was developed that incorporated the capability to identify neighbours present in each of the amino acid residues in an enzyme. Such an analysis on members of the cytochrome family, separated by aeons in the evolutionary scale, showed uniform departure from values expected if there were no preference in the choice of a neighbour (TABLE S.2 - S.18 = TABLE C.2 - C.18). This conclusion was confirmed by a similar analysis of the 500 residue peptide, bovine glutamate dehydrogenase (TABLE S.19 = TABLE C.19). The persistent departure from the anticipated "no preference values (R.V.)" in the choice of neighbour further supports the existence of inherent preference in the selection of neighbours.

The diagonal element in each of the above tables denotes preferences for having as neighbour another unit of the same amino acid. In TABLE S.2 - S.18 = TABLE C.2 - C.18, the diagonal element is zero in 75-80% of the cases, which would indicate a lack of preference for itself, barring few exceptions. Even in the case of the larger protein presented in TABLE S.19 = TABLE C.19, 50% of the diagonal element is zero. There is every reason to believe that this would be the case in more extensive analyses planned with a much larger data base.

For the cytochrome family, preferences for neighbours of value 3 and above have been processed from TABLE S.2 - S.18 = TABLE C.2 - C.18 and summarized in TABLE S.21 = TABLE C.21. A striking feature is the nearly consistent preference exhibited by lysine residues to have another lysine as neighbour. Since the coverage involves a wide time span in terms of evolution,

it is logical to conclude that preferences with respect to the choice of a neighbour was operational from very early stages of protein evolution.

The Ser-Ser unit was selected for detailed analysis because of its apparent rarity in spite of the fact that the Ser residue is relatively abundant in proteins. This notion was confirmed by an analysis of 150 proteins involving diverse enzyme activity. Barring notable exceptions, the observed Ser-Ser values were either zero or much below the random values (R.V.). In spite of the observed general rarity of the Ser-Ser combination, in few they are abundant ; further, this abundancy is typical and can be correlated to specific proteins such as carbonic anhydrase, the proteases trypsin, pepsin, subtilisin and carboxypeptidase, the trypsin inhibitors, elastase, ribonuclease and deoxyribonuclease. Thus, nucleases and proteases harbour excessive Ser-Ser combinations. Additionally, the presence, of 5 Ser residues in a row in ribonucleases and 4 Ser residues in subtilisin, must be considered significant.

In sum, experimental and analytical results strongly support the existence of preferences in the choice of neighbour which, in turn, tend to support a convergent approach to protein evolution (CHART S.10=CHART C.11).

In the work described thus far, polar interactions were a major consideration in achieving selectivity in peptide bond formation. The preponderance of hydrophobic residues in proteins points to environments conducive for preferential peptide bond formation involving hydrophobic residues. In the present work, it was envisaged that if amino acids having hydrophobic side chains could be aligned with an appropriate condensing agent on a micellar support, peptide bond formation can occur at water interface leading to preferential formation of peptides having hydrophobic amino acid residues.

The micellar support chosen in the present study comprised of the isooctane - bis(2-ethyl hexyl)sodium sulfosuccinate (AOT) - water system whose profile has been established as illustrated in CHART S.15 = CHART C.17. The strategy called for the condensing agent in the role of the co-surfactant. To this end, the novel dioctadecyl carbodiimide (DODCI) (39), was prepared, tested for its effectiveness in the formation of peptide bonds, and subsequently used to generate the interface support, illustrated in CHART S.16 = CHART C.21.

Two possible situations can arise when coded α-amino acids are added to the DODCI - AOT reverse micelle composite (CHART S.16 = CHART C.21). In the case of α-amino acids possessing hydrophobic residues, the side chains can be expected to be a part of the micellar surface with the α-amino acid moiety facing the water-pool. Such an arrangement, illustrated in CHART S.17a = CHART C.22a, would lead to the activated ester followed by peptide bond formation. On the other hand, in the case of amino acids carrying polar side chains, they could be expected to be present mostly in the water pool and the peptide bond formation involving these compounds would be governed by their interaction with DODCI anchored at the micellar interface CHART S.17b = CHART C.22b.

Peptide bond formation was achieved using the micellar support illustrated in CHART S.16 = CHART C.21 with a number of amino acid residues having hydrophobic as well as polar side chains. The results, as shown in CHART S.18 = CHART C.23 is good and the sole exception involving proline could be understood on the basis of the ability of this imino acid to align at the micellar interface on the one hand, and the high solubility arising from sequesteration of the hydrophobic side chains on the other. The preference for peptide

bond formation involving hydrophobic residues was further established in a competitive environment. Thus, N,γ -C-protected glutamic acid and N,β -C-protected aspartic acid selectively formed peptide bonds with LeuOMe in preference to GludiOMe/Asp-diOMe.

In the present work, selectivity, taking advantage of order that exists either in aqueous or in micellar media with reference to peptide bond formation has been established.

CONTENTS

* * * *

A. INTRODUCTION

Each and everyone of the multifaceted manifestations of Nature mirrors, above all, the consequences of evolution. If this indeed is true in the macroscopic world that we are able to perceive, logic dictates that such must be the case at the microscopic level involving molecular interactions. The focus of the present work is directed at the possible understanding of the functional system, encompassing the most complex of molecular constellations arising from a carefully and methodically orchestrated aggrandization of 20 coded α-amino acids. The concatanation of the latter, gives rise to the primary sequence, which is coded in the information system.

From the error free replication and transcription involving Nucleic acids to the synthesis of the very primary unit, namely, the α-amino acid, the functional system - mostly synonymus with enzymes - controls all the molecular events that are necessary. There is universal agreement to the effect that the enzymes themselves must have evolved through various stages, the very early ones necessarily via chemical processes. It appears logical, therefore, that a good deal of order must have existed before the advent of present day enzymes. An enquiry into these early developments should have, as focus, selective peptide bond formation on the one hand and the recognition of preferred peptide segments on the other, since such synthons are most likely to be involved in the further condensation to more complex systems. Both these aspects have been experimentally probed in the present work (SECTION C.).

In the present work, selectivity in peptide bond formation involving coded α-amino acids has been sought taking advantage of either intrinsic order

in the crystal lattice or the possible arrangement in aqueous media or at micellar interface. It was, therefore, considered appropriate to present a brief background pertaining to each of the above facets. This has been done in the following section (SECTION B.).

B. BACKGROUND

THE ORDERING OF AMINO ACID COMPOSITES IN A CRYSTAL LATTICE.

Non-covalent interactions involving amino acid residues play a crucial role in the structure, assembly and function of proteins. An interesting, common feature present in most amino acids that have been subjected to X-ray analysis is the aggregation of amino acid molecules in a head to tail sequence (CHART B.I.)[1]. In this preferred arrangement, the α-amino and the α-carboxylic groups are brought into periodic hydrogen bonded proximity in a peptide like arrangement, an arrangement that could have possible relevance in the formation of non-enzymatic formation of polypeptides.[2]

An arrangement such as shown in CHART B.I does not account for the influence of the side chains in peptide bond formation. A number of experiments have been carried out to demonstrate non-enzymatic synthesis of polypeptides.[3-8] Many of these interesting experiments have clearly established that the polypeptides thus formed have non-random sequences, thus, pointing to the possibility of self odering of amino acids.

Although, it is logical to assume an intrinsic ordering of diverse amino acid residues incorporating preference for neighbours in the formation of peptides, the delineation of such preferences poses experimental complexities.

A clear approach to this problem envisaged the preparation of mixed crystals with amino acid pairs and establishment of their structures

CHART B-1

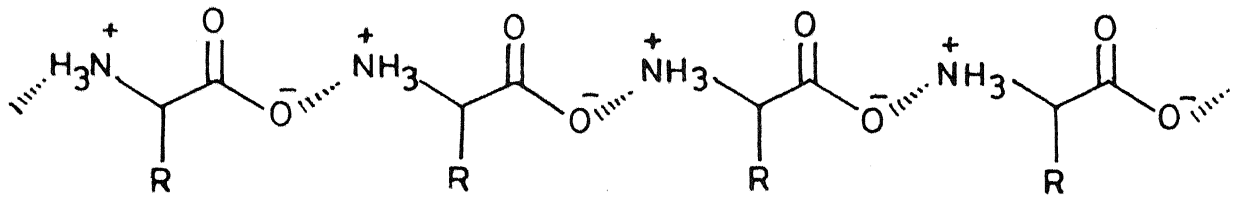
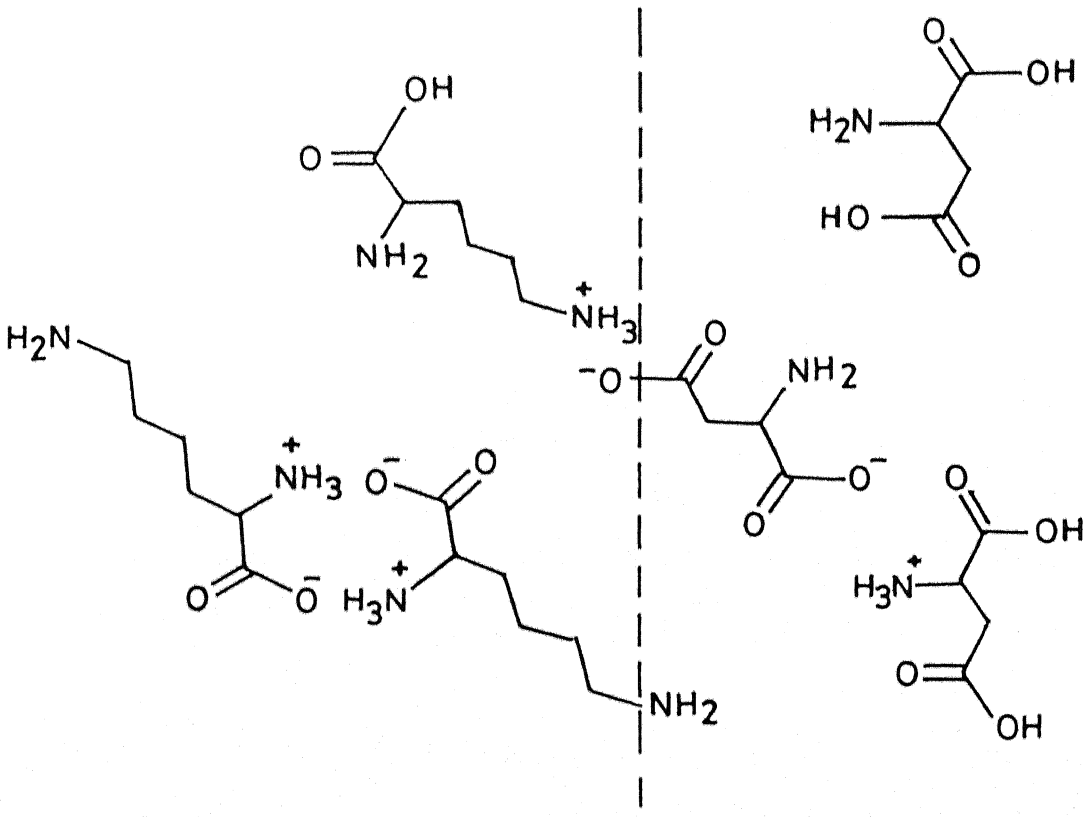


CHART B-2

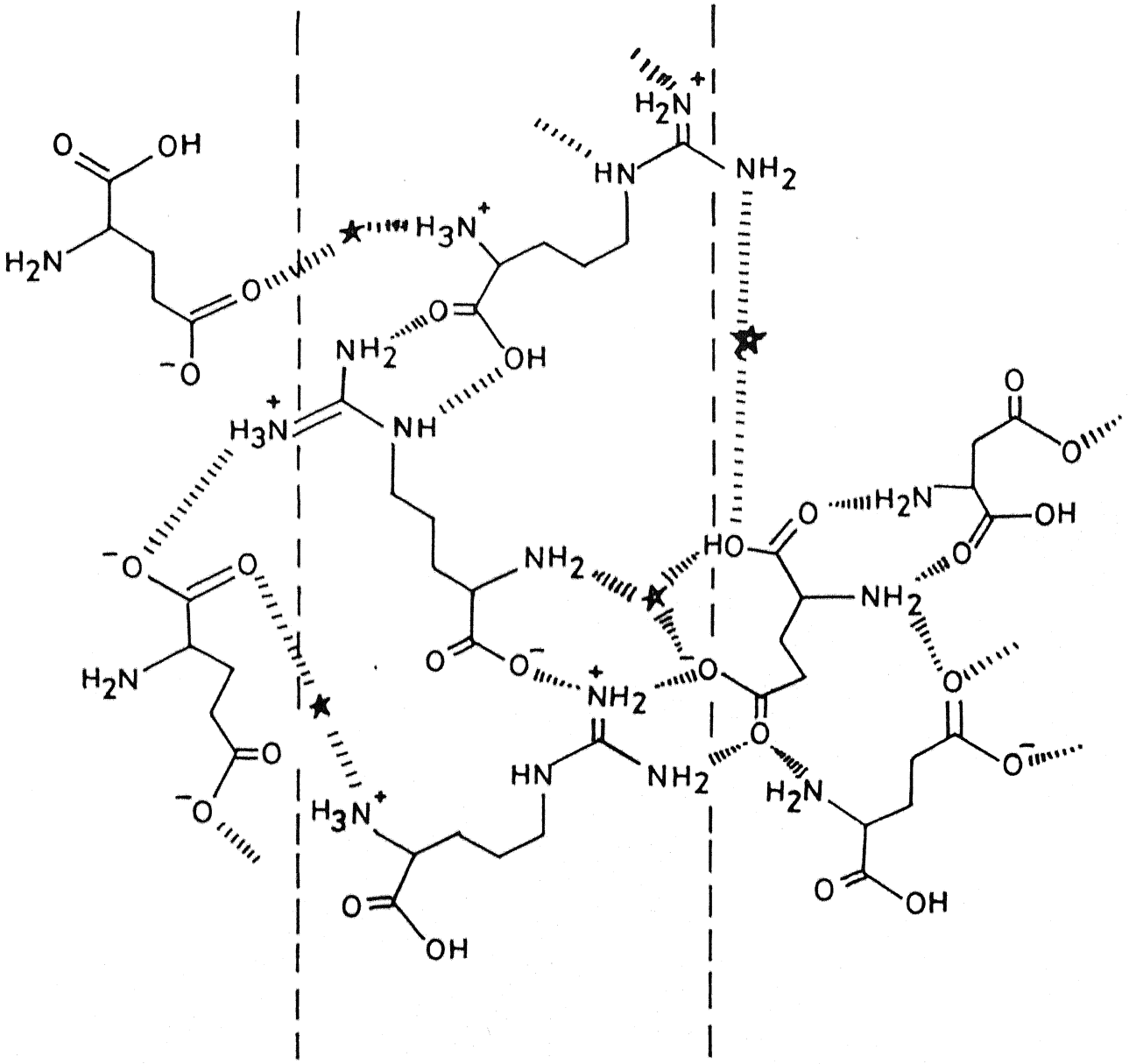


by X-ray crystallography. Such studies, although not uniform in all cases clearly established preferences for alignment.

Crystals of lysine and aspartic acid were grown by slow evaporation at room temperature of an aqueous solution leading to crystals which comprise of equimolar quantities of both the components. The X-ray analysis of these mixed crystals consisted of alternating layers, one layer comprising of lysine molecules and the other aspartate ions. A profile of such an arrangement is illustrated in CHART B.2 which for clarity has been confined to two layers. As could be clearly seen from CHART B.2 both lysine as well as aspartic acid retain the head to tail arrangement of the α-amino acid moiety. At the same time a point of significance is that the crystal structure favours the peptide bond formation involving the ω-amino group of lysine with the β-carboxyl group of aspartic acid. This pattern appears to hold good in several cases. Thus, whilst self condensation would lead to normal peptide bond formation, cross condensation could be predicted to the formation of peptide bond involving side chain residues.[9]

A somewhat more complex situation arises with mixed crystals involving glutamic acid and arginine. A few tiny crystals of this complex analysing for glutamic acid - arginine - H_2O could be grown after carefully controlled and repeated attempts by the slow diffusion of acetone into an aqueous solution of the mixture. The X-ray results of these mixed crystals are represented in simplified form in CHART B.3. The crystal structure consists of alternating layers, one layer containing arginine and the other glutamic acid. As could be seen from CHART B.3 similar residues preserve a head to tail arrangement,

CHART B-3



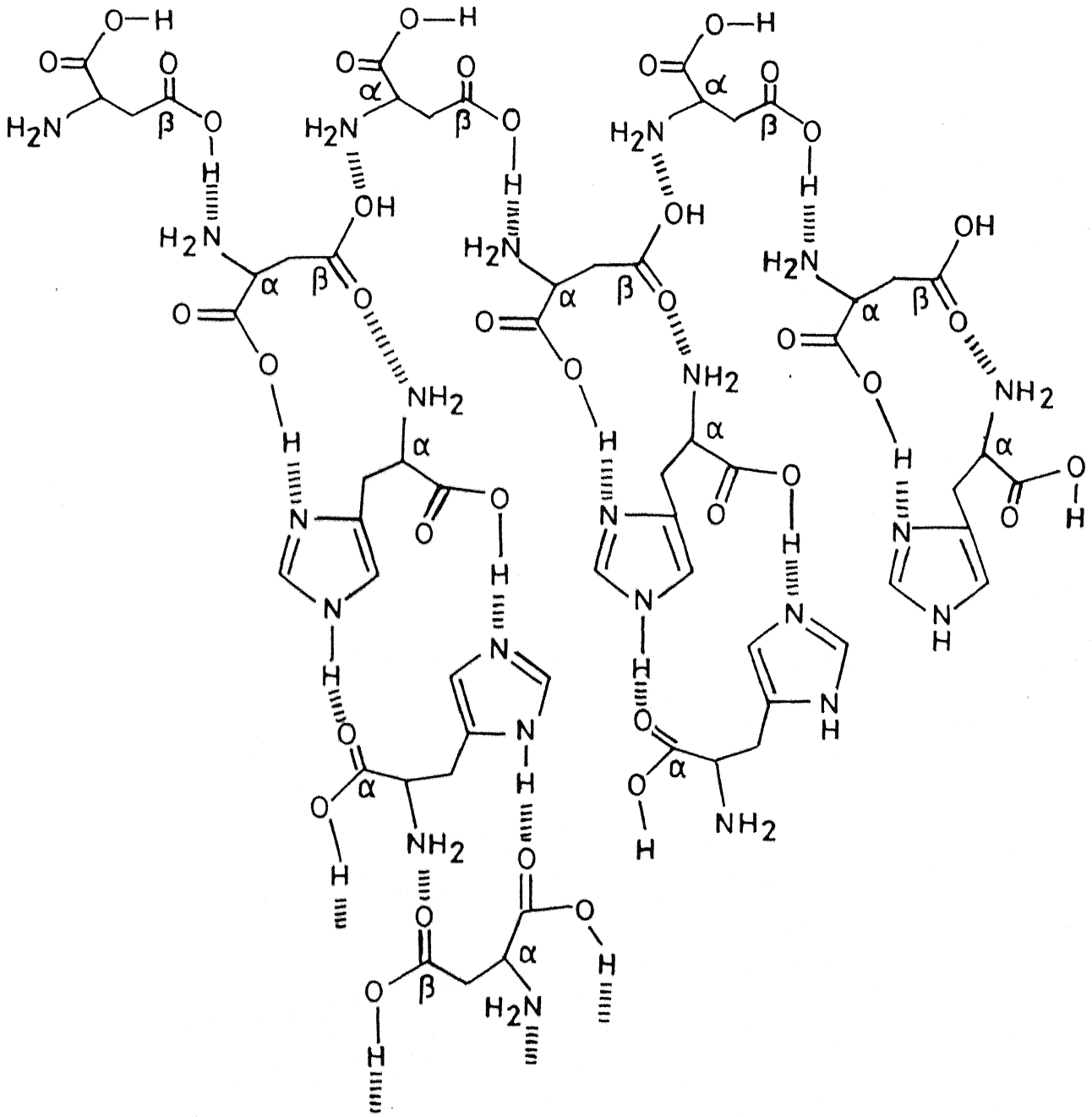
thus condensation in this fashion could lead to polyglutamic acid. However, the products of cross condensation are difficult to predict and one possibility certainly is the condensation of the α -amino acid of the arginine to the γ -carboxyl group of the glutamic acid.[10]

Equimolar amounts of histidine and aspartic acid in water on slow diffusion of acetone leads to needle -like crystals which analyse for histidine - aspartic acid - water. In this particular case, the crystal structure consisted of alternating double layers, one double layer containing histidine molecules, the other aspartic acid molecules. The water molecules are sandwiched between the two layers in the aspartic acid double layer (CHART B.4 = CHART C.1). In view of the direct correlation of this to the present work the implications of this arrangement is discussed in SECTION C.[11]

An extremely interesting observation which emerged from studies of such mixed crystals is that even in the case of mixed crystals involving dipeptides, order is preserved in the crystal lattice. Thus, equimolar amounts of L-Histidyl-L-Serine and L-Glycyl-L-Glutamic acid in water on slow evaporation crystallises giving rise to histidyl serine - glycyl glutamic acid - 6 H_2O. Here again, the like molecules aggregate into separating alternate layers. A cross section of this arrangement is shown in CHART B.5.[12]

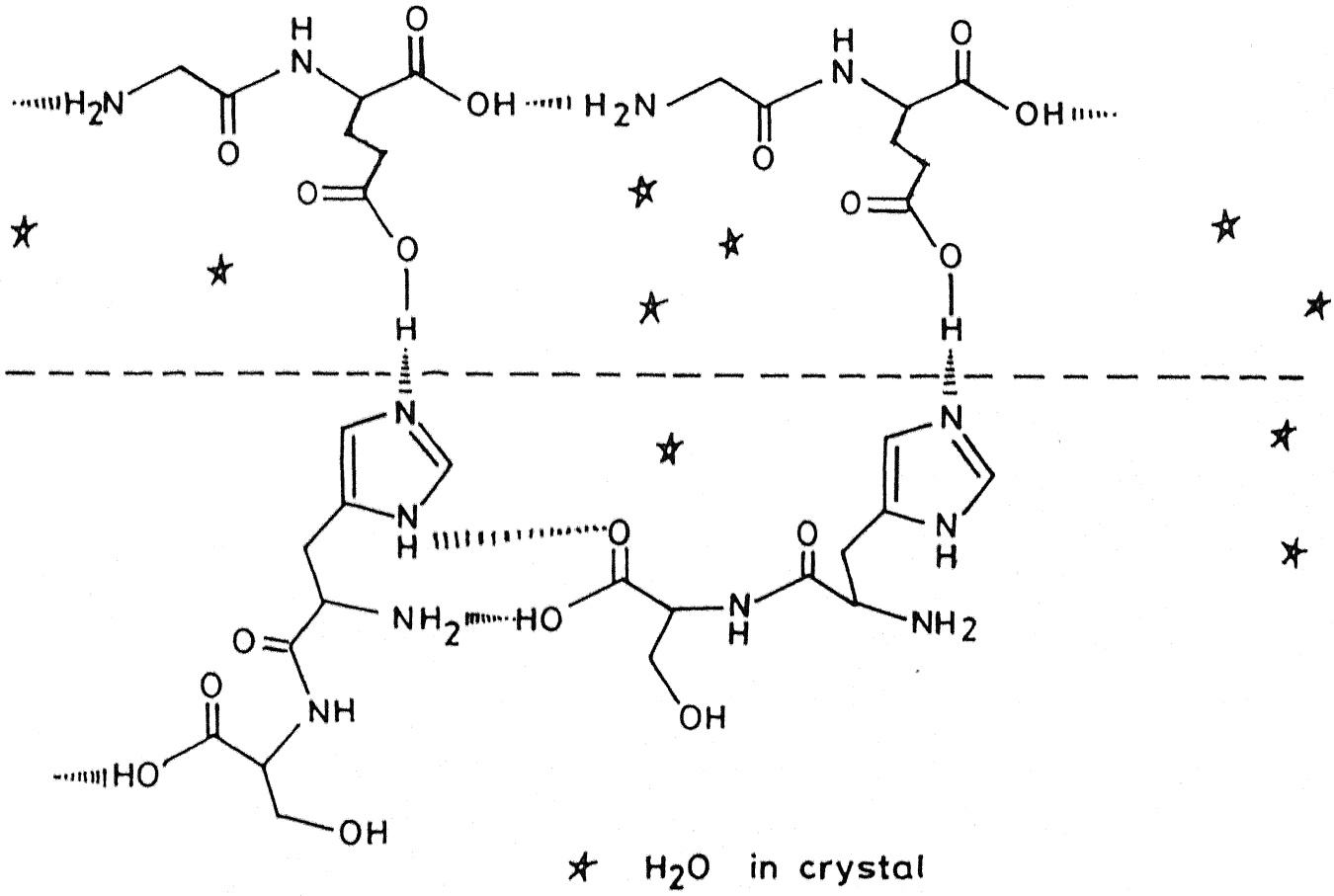
In the event, peptide bond formation takes place involving similar residues, it could be clearly seen from CHART B.5, the sequence would be Gly-Glu(γ -OH)-Gly-Glu(γ -OH) .... or Ser-His-Ser-His... The arrangement of the crystal is such that it will be difficult to predict the nature of cross condensation product.

CHART B-4



ORDER IN ASP-HIS MIXED CRYSTAL

CHART B-5



The brief account pertaining to a discussion of results obtained from X-ray crystallography makes it very logical to assume that molecular order would prevail in the crystal lattice irrespective of whether monomeric residues or polypeptides are used. Peptide bond formation, taking advantage of such an arrangement would certainly lead to preferences. However, to achieve this, serious experimental impediments have to be overcome and an aspect of the present work (SECTION C.) endeavoured to achieve this objective.

WATER SOLUBLE CARBODIIMIDES

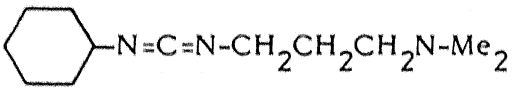
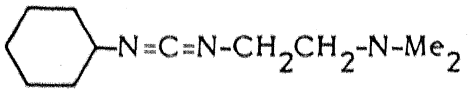
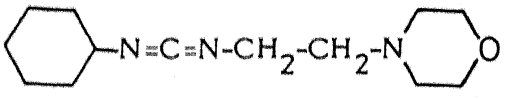
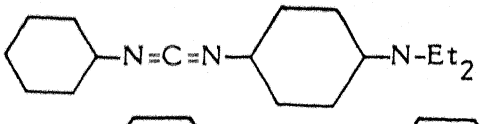
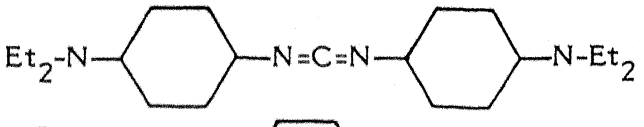
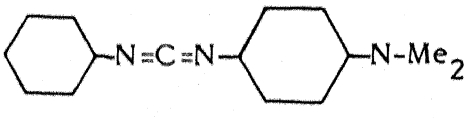
Initially, the water soluble carbodiimides came into vogue to obviate a persistent problem in peptide synthesis using DCC namely, the removal of the DCU formed in the reaction which is soluble in common organic solvents used in peptide synthesis. It was considered that a urea derivative that is either water soluble or could be removed by treatment with dilute aqueous acids would eliminate the problem of urea contamination in peptides.

The commonly used water soluble carbodiimides are presented in CHART B.6. In most cases, they are used as quaternary salts because of the enhanced stability of such compounds. Over a period of time, the importance of water soluble carbodiimides in peptide synthesis is finding increasing appreciation particularly with reference to the preparation of catalytic antibodies (vide infra).

In the initial phases of study involving water soluble carbodiimides, the peptide bond was generated in organic solvents using the hydrochloride of I(CHART B.6). Thus, oligopeptides without isolating intermediates wereprepared even retaining troublesome residues like ser, thr, met and his. The first

CHART B-6

COMMONLY USED WATER SOLUBLE CARBODIIMIDES

No.	STRUCTURE	REF.
I.	$CH_3CH_2N{=}C{=}N\text{-}CH_2CH_2CH_2N\text{-}Me_2$	13, 14, 15, 16, 17.
II.	$PhCH_2N{=}C{=}N\text{-}CH_2CH_2CH_2N\text{-}Me_2$	18
III.	$CH_3CH_2N{=}C{=}N\text{-}CH_2\text{-}CH_2\text{-}N$ 	19, 20
IV.		21, 22
V.		22
VI.		22, 23, 24, 25.
VII.		22
VIII.		22
IX.		22

significant achievement was the synthesis of an insulin segment by this procedure.[13]

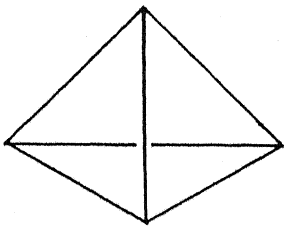
Recently, urgasterone - human epidermal growth factor-was synthesized by the segment condensation method. In this noteworthy achievement ten smaller segments were sequentially condensed using I in presence of HOBt, ultimately resulting in the preparation of the pure 53 residue peptide.[14]

Most recently, an entirely new concept in the design of biological catalyst has emerged, whose underlying principle rests on the fact that a receptor or an active site designed to optimally bind a suitable analog of a transition state would achieve the catalytic function. In an ingenious application of this concept the 4-nitrophenyl phosphonate X,(CHART B.7) was attached to bovine serum albumin (BSA) using the water soluble carbodiimide I, which was further processed to monoclonal IgG that was demonstrated to efficiently catalyse the hydrolysis of diverse carbonyl substrates with high degree of specificity. The cardinal principle in this experiment is that compound X could be considered as an appropriate transition state for the hydrolysis of carbonates and consequently, antibodies raised against this should catalyse the hydrolysis.[16]

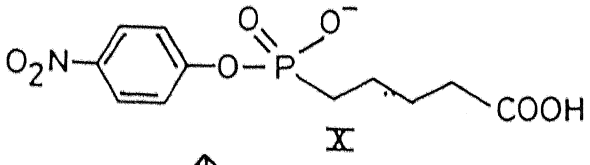
The interesting cyclic hexapeptide XI(CHART B.8) was synthesised principally to study the interaction of the histidine and tyrosine side chains with each other, with solvents and with other molecules. The synthetic strategy as shown in CHART B.8 extensively used I as the condensing agent to obtain high yields of the pure compound.[17]

The water soluble carbodiimide II has been used to bring about the Lossen rearrangement of hydroxamic acids(CHART B.9).[18]

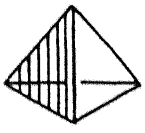
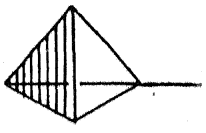
CHART B-7

a. SUBSTRATE ⟶  ⟶ PRODUCT

TRANSITION STATE



X

b.  —BOVINE SERUM ALBUMIN (BSA)→ 

WATER SOLUBLE CARBODIIMIDE

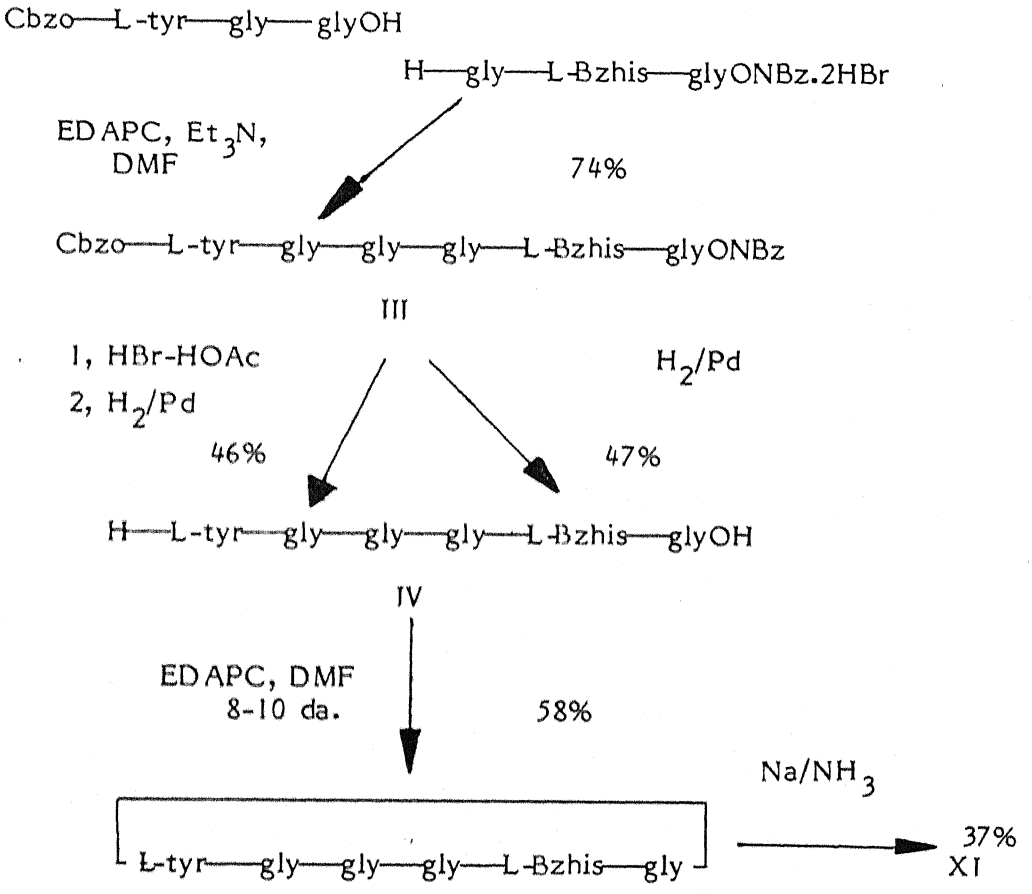
STABLE TRANSITION STATE MODEL

FUSION WITH sp 2/o MYELOMA CELLS ⟶ IgG (RECOGNIZES TRANSITION STATE)

c. SUBSTRATE ⟶ PRODUCT

IgG(ABZYME) CATALYSIS

CHART B.8



V.

Synthesis of the cyclic hexapeptide; abbreviations : Bzhis, N^{im}-benzylhistidine; Cbzo, carbobenzoxy; NBz, p-nitrobenzyl; DMF, dimethylformamide; EDAPC, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide.

CHART B-9

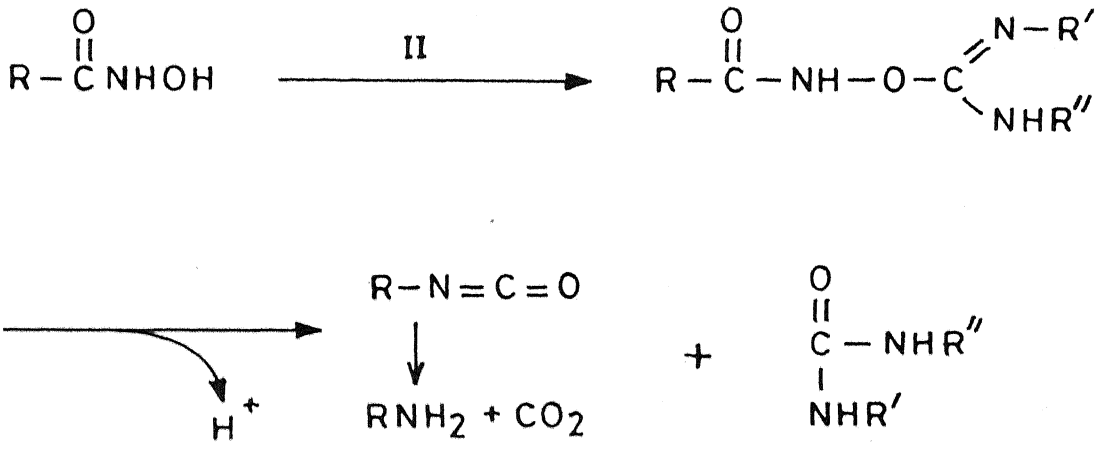
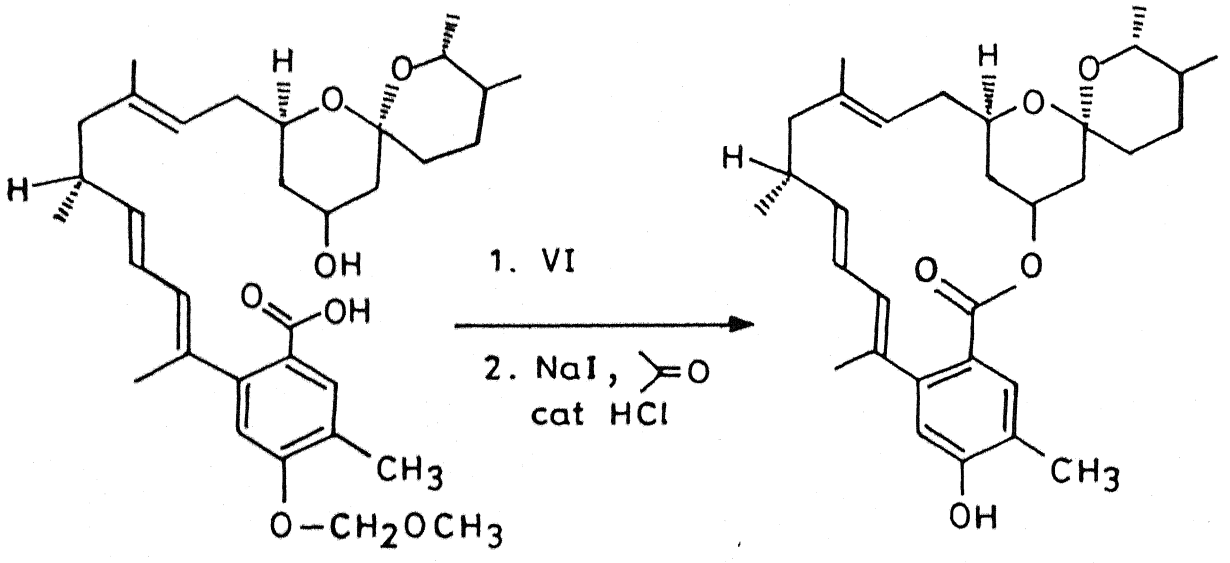


CHART B-10



An unusual application of water soluble carbodiimides III is to effect the cross-linking of side chain groupings in the water soluble protein gelatin thus, greatly enhancing its propensity to form gels.[19]

The water soluble carbodiimides III and VI have been used to study the degree of polymerisation of glycine as a function of concentration of substrate, the amount of the reagent employed and the pH of the medium. The results of this interesting study is presented in TABLE B.1.[20]

Compounds such as VI could promote in refluxing organic solvents ready formation of azlactones as exemplified with benzoyl glycine.[24]

Apart from their obvious use in peptide synthesis, water soluble carbodiimides have found application in bringing about delicate transformations. This is exemplified with a very efficient synthesis of the antibiotic, milbemycin β_3 (CHART B-10).

The water soluble carbodiimides listed in CHART B.6 can be used either as such or as simple hydrochlorides or equivalent salts or as quaternary salts arising from transmethylation involving paratoluene sulfonic acid methyl ester.

In the present work, the water soluble carbodiimides have been used with a novel objective, namely, to discern preferences in the formation of peptide bonds from amongst several possibilities (SECTION C.).

TABLE B-I

Glycine Concentration %	Glycine/ reagent	pH	Unreacted Gly	di-pep tri-	Tetra - ↓ hexa	Poly - mer
20	1	3	3	30	50	2
20	1	7	30	3	3	0
20	2	4	30	40	15	0
20	0.5	6	1	5	15	17

REVERSE MICELLES

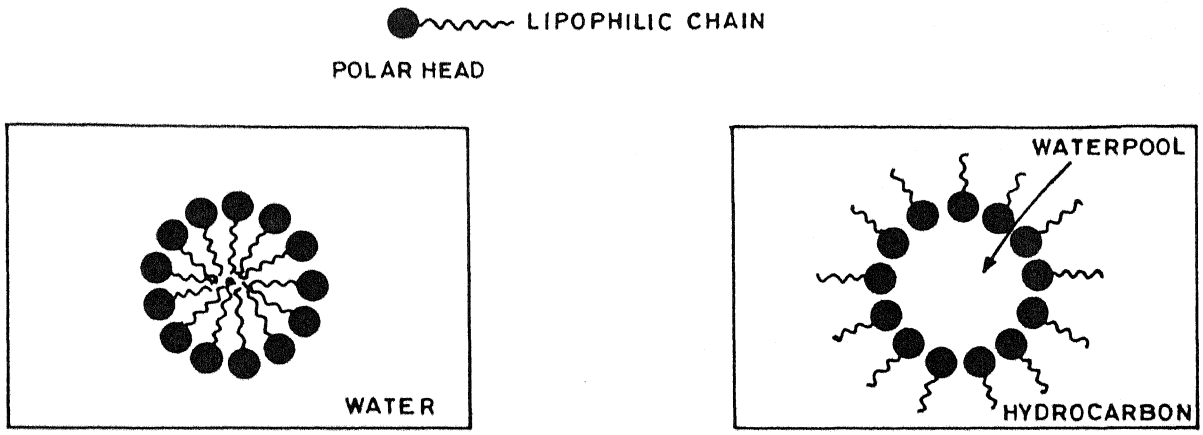
Reverse micelles are ordered assemblies that are formed when some surfactants are dissolved in non polar solvents. Since the polar heads of the surfactant naturally tend to avoid the solvent, they form a polar core with the hydrophobic chain engulfed in the solvent. Interestingly, the nomenclature of such systems as reverse micelles arose from the fact that historically the reaction of surfactants in water was long recognized and studied and in these systems the ordered assembly arising from aggrandization of the hydrophobic tails in the core with the polar heads directed at water, was termed a micelle (CHART B.11).

The great current interest in reverse micelles is primarily because of their ability to harbour water pools of varying properties. Indeed, such systems are considered as ideal subjects of an investigation for those chemists who are interested in the spontaneous formation of ordered structures.[26]

Properly prepared reverse micelles systems in non polar solvents are clear and enable the study of water soluble systems ranging from simple molecules like polyhydroxy compounds to the most complex enzymes and nucleic acids by spectroscopic studies. The properties of such systems present in the water pools of the reverse micelles, are similar in most cases to that in bulk water including enzyme activity.[27-29]

Perhaps the most widely used surfactant in the preparation of reverse micelles is Aerosol-OT(AOT) which is bis-(2-ethylhexyl)sodium sulfosuccinate (XI). In the present work also the reverse micellar system study involves AOT (SECTION C.).

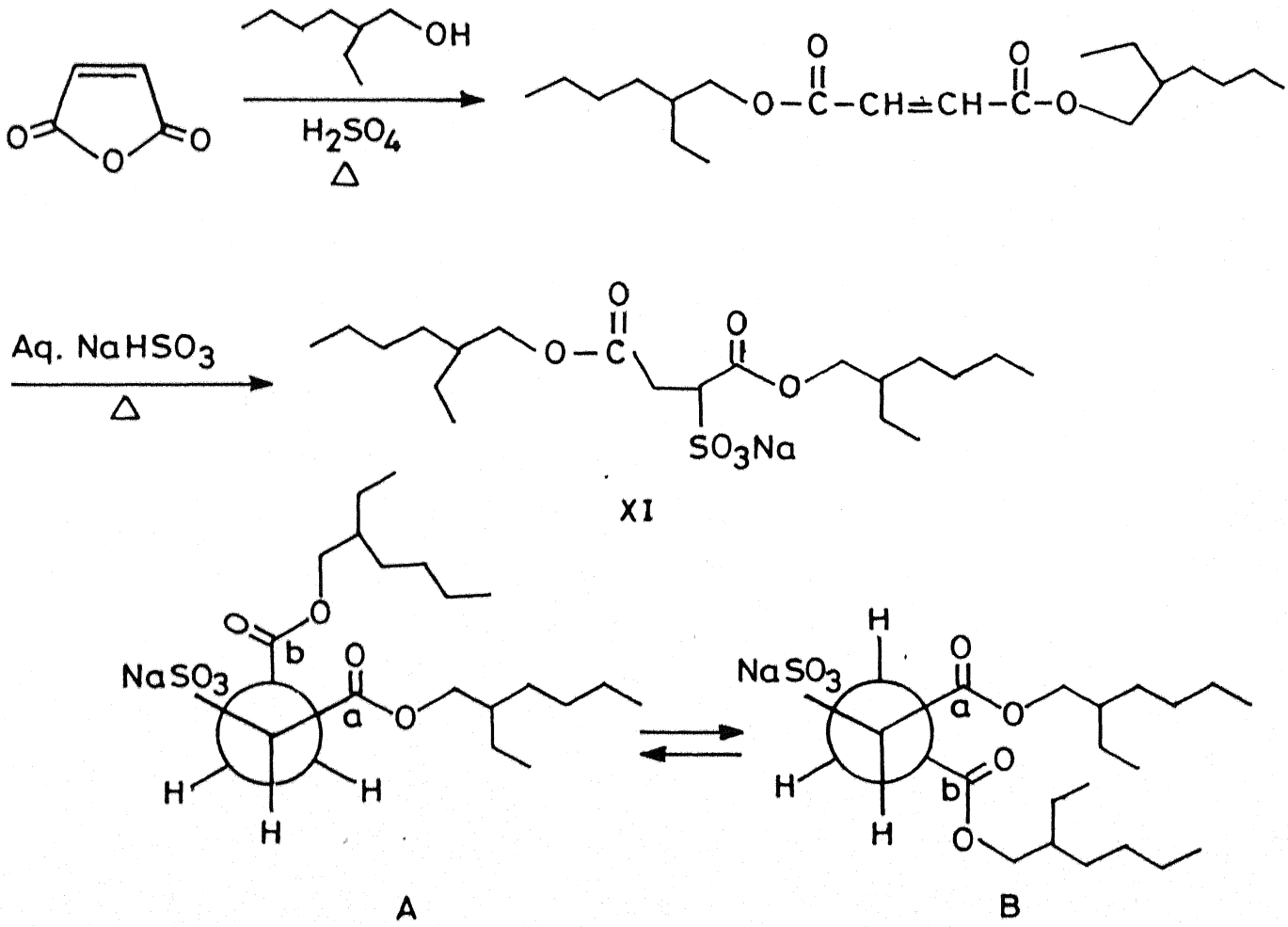
CHART B-11



Normal micelles

Reverse micelles

CHART B-12



The other names for AOT are, Sodium dioctyl sulfosuccinate ; DSS ; AlphasolOT ; Colace ; Comfolax ; Complemix ; Coprol ; Dioctylal ; Dioctyl-Medo Forte ; Diotilan ; Diovac ; Disonate ; Doxinate ; Doxol ; Dulsivac ; Molatoc ; Molcer ; Molofac ; Nevax ; Norval ; Regutol ; Softil ; Soliwax ; Solusol ; Sulfimel ; DOS ; ValsolOT ; Velmol ; Waxsol.

In view of the importance of AOT, its preparation and general properties are noteworthy. AOT is prepared via opening of maleic anhydride with 2-ethylhexanol followed by Fischer esterification of the resulting carboxylic acid with the same alcohol. Michael addition of aqueous sodiumbisulfite to the resulting fumarate leads to AOT (CHART B.12).

An important parameter that defines the nature of water pools in reverse micellar systems involving XI is $w_o = [H_2O]/[AOT]$.

The important rotomers of XI are shown in CHART B.12. It has been observed by ^1H and ^{13}C nmr that as w_o increases in isooctane, the conformational equilibrium shifts towards B. It could be noted that in rotomer B the side chain designated as (b) moves deeper into the interface thus creating an enhanced micellar order (CHART B.12).

An astonishing property of AOT is that, it is soluble in every solvent ranging from water to hydrocarbons. Commercial AOT preparation should be checked to ensure absence of impurity and products arising on hydrolysis, since these contaminants could affect reactions involving sensitive enzyme systems.

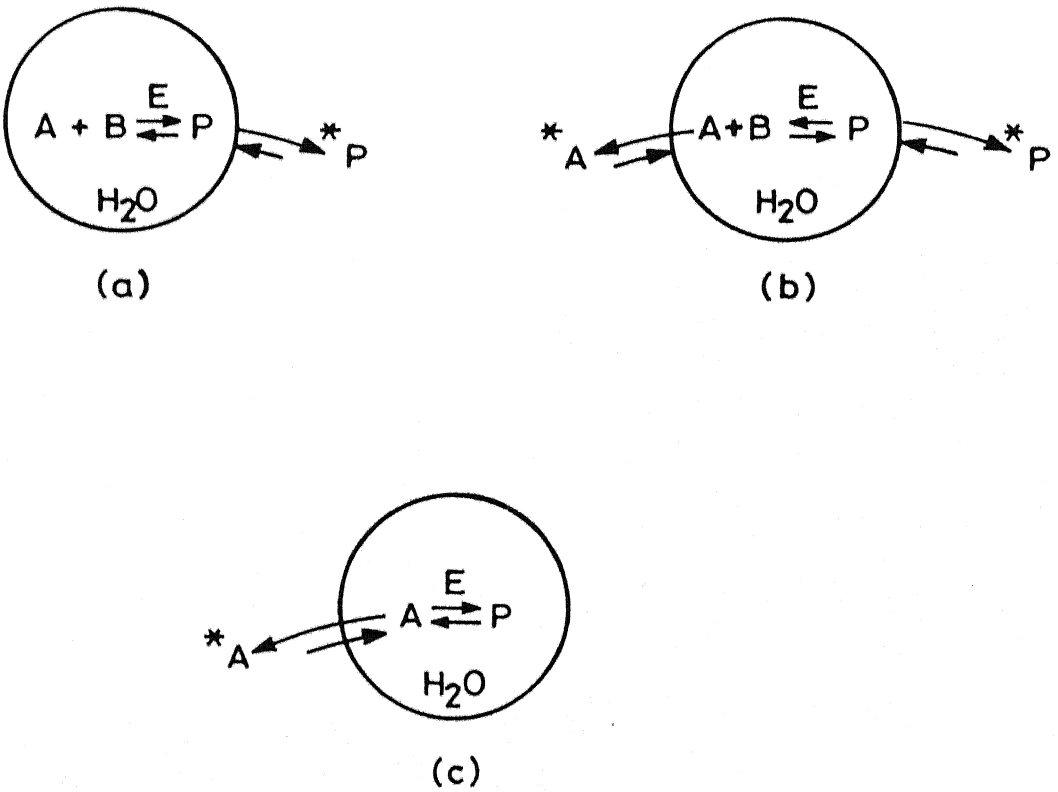
The importance of $w_o = [H_2O]/[AOT]$ with respect to AOT alignment has been referred to earlier. The quantity w_o also determines the nature of water associated with AOT reverse micelles in isooctane. Extensive experi-

mentation led to the conclusion that at values for w_o in the range from 6-8, the water molecules are tightly bound to the head of the surfactant. In the range where w_o is 8-16 represents a true reverse micellar situation incorporating trapped water whose rotational motion is hindered, whose freezing point is significantly depressed and a state that has been used for demonstration of greatest reactivity. Beyond $w_o > 20$ the system becomes a microemulsion with the water exhibiting near normal properties.

Reverse micelles behave like membrane vesicles in many ways and the dynamic nature in solvents enable them to coalesce into larger structures which in turn can also break down into smaller units. Thus, in a truly dynamic situation, the concentration of diverse materials in a reverse micelle could attain equilibrium concentration. Much of the interest involving AOT reverse micelles and other equivalent systems, is in the use in microreactors. Diverse scenarios involving the permutations of the substrates, the reagent and the product could be constructed to make the operation highly versatile. This aspect is illustrated in CHART B.13 which represents the union of substrates A and B to form the product, in this particular case using a water soluble enzyme as a catalyst.[31] In illustration CHART B.13a, the reagents A and B are preferentially soluble in water but the product P is not. Consequently, the products formed would be dispelled from the microreactor environment thus making the process continuous. CHART B.13b represents a situation where A is largely insoluble in water as well as the product. In this situation, the diverse equilibria that exist would ensure the continuous transformation of the water insoluble A to the water insoluble product P. In CHART B.13c, whilst the substrate A is largely insoluble in water, the product P is and in the existing equilibria would ensure the accumulation of the product in

CHART B-13

ISOOCTANE



the water pool.

Thus, the reverse micelles as microreactors permit interesting chemical reactions when the products and the reactants have opposite solubility characteristics.

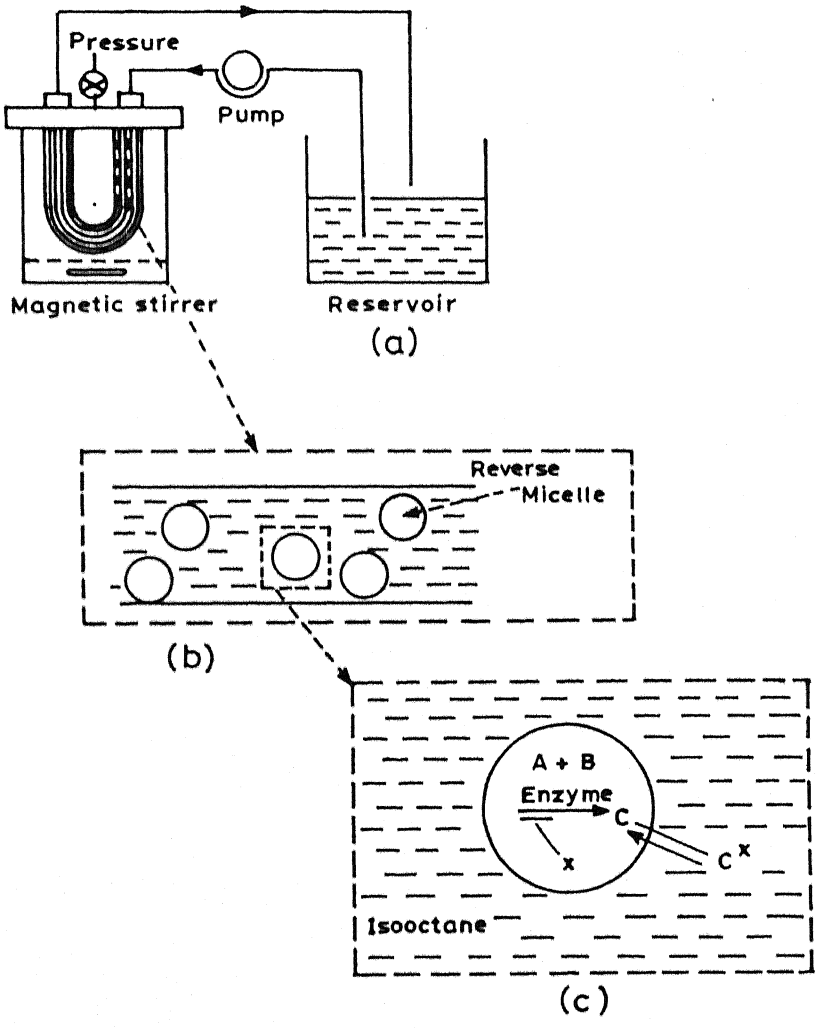
In view of the importance of the efficiency with which the usually insoluble product has to be removed from the microreactor environment, a spatial segregation of the product is desirable. An ingenious approach to this problem which has profound relevance in biotechnology uses a reactor design presented in CHART B.14 which ensures the segregation of the reagent / catalyst (enzyme) from the product. The U tube arrangement in CHART B.14 consists of semipermeable tubular fibres constructed using inert polyamides which prevent the enzymes containing the reverse micelles to enter the bulk hydrocarbon interface. In CHART B.14 the enlarged profile of the tubules are shown in (a , b , and c).[32] The operation of this reactor involves the application of the enzyme catalyst as well as the substrates using a microsyringe inside the hollow fibre and harvesting the product from the bulk isooctane. This reactor has been used for the preparation of Z-Ala-Phe-Leu-NH_2 and Ac-Phe-Leu-NH_2.

$$\underset{A}{\text{Z-Ala-Phe-OMe}} + \underset{B}{\text{H-Leu-NH}_2} \overset{E}{\rightleftharpoons} \underset{C}{\text{Z-Ala-Phe-Leu-NH}_2} + \text{MeOH}$$

$$\underset{A}{\text{Ac-Phe-OEt}} + \underset{B}{\text{H-Leu-NH}_2} \overset{E}{\rightleftharpoons} \underset{C}{\text{Ac-Phe-Leu-NH}_2} + \text{EtOH}$$

Although the yields are not spectacular, the design represented in CHART B.14 is indeed a fore-runner of many important developments that are bound to take place in this domain.

CHART B-14


Pressure
Pump
Magnetic stirrer
Reservoir
(a)
Reverse
Micelle
(b)
A + B
Enzyme
C
x
C x
Isooctane
(c)

The diverse application involving AOT or equivalent reverse micelles are summarized below.

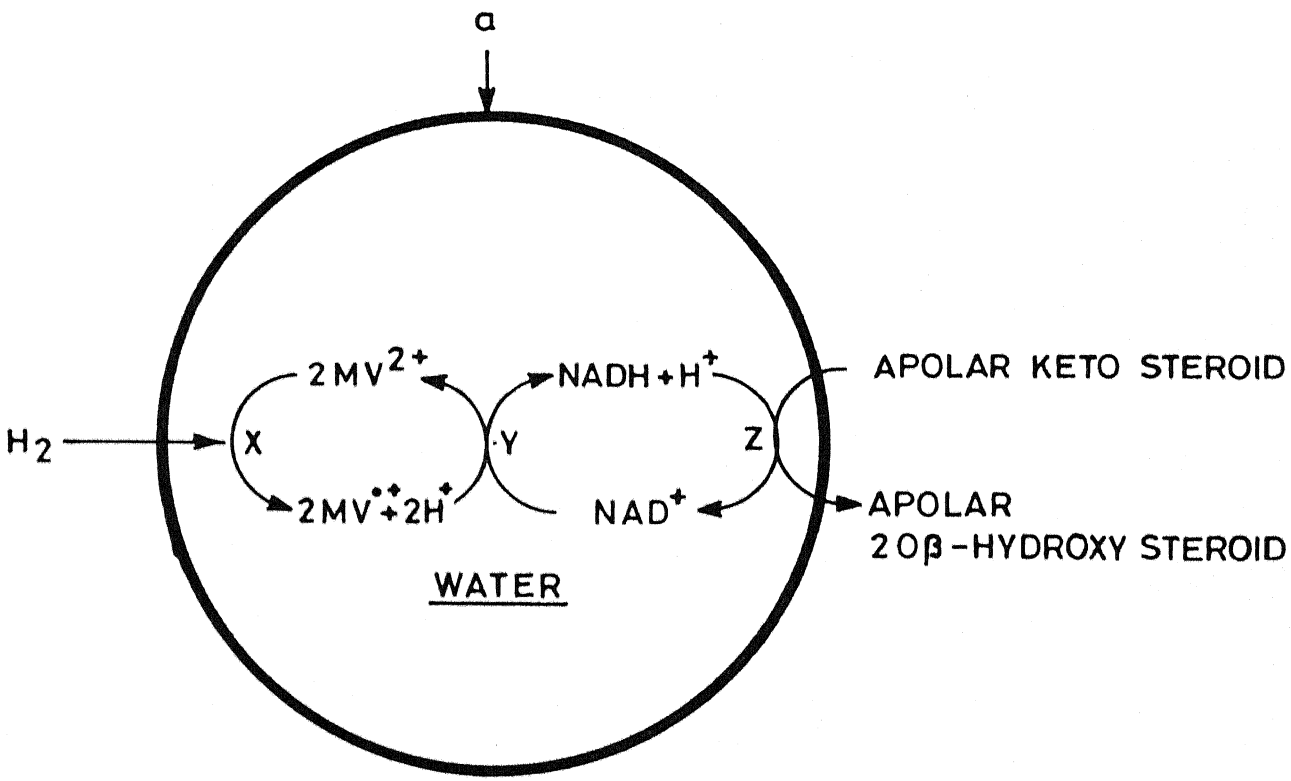
The oxidation of poly unsaturated acids with molecular oxygen using oxygenase enzymes are very important biological processess pertaining to the metabolism of these edible substances. Reliable kinetics pertaining to this oxidation were hard to obtain because of poor solubility of the substrate. Reverse micellar systems involving AOT provided a admirable solution to this problem.[33]

Steroids in general are hydrophobic and their enzyme mediated transformation poses problems in the laboratory. Here again, the reverse micellar systems provides a useful microreactor. Thus, using AOT isooctane system, ketosteroids have been reduced using the appropriate co-enzyme - enzyme combination. In a similar manner progesterone and prednisone have been reduced using the appropriate enzyme in combination with NADH coenzyme using cetyltrimethyl ammoniumbromide (CTAB)/ hydrocarbon reverse micelle system. In this reaction, the NADH was regenerated by hydrogenase that uses hydrogen. Thus, the gaseous hydrogen reduces methyl violegen (MV^{++}) which in turn reduces NAD^{+}. Thus NAD^{+} and MV^{++} are recycled whilst hydrogen is consumed (CHART B.15).

The above examples cited are illustrative and by no means exhaustive.

The use of immobilized enzymes currently play a pivotal role in biotechnology. Whilst the reverse micellar systems currently represent a very early stage in development, this technique has potential to even surpass immobilized metholodolgy in terms of simplicity, costs and versatility. Micellization

CHART B-15



a = CTAB / alcohol surfactant

X = Hydrogenase, Y = Lipoamide dehydrogenase

Z = Steroid dehydrogenase

MV = Methyl viologen

of enzymes affords flexibility in terms of reaction parameters. The great flexibility here permits the use of a whole range of a polar substances. However, much remains to be done in the development of appropriate technologies to enable optimization for parameters.[34]

In the present work, the reverse micellar system has been used in a novel manner to achieve selectivity in peptide synthesis. In this, the peptide bond formation is envisaged to take place at the micellar interface mediated by an appropriate cosurfactant carbodiimide.

SECTION C : PRESENT WORK

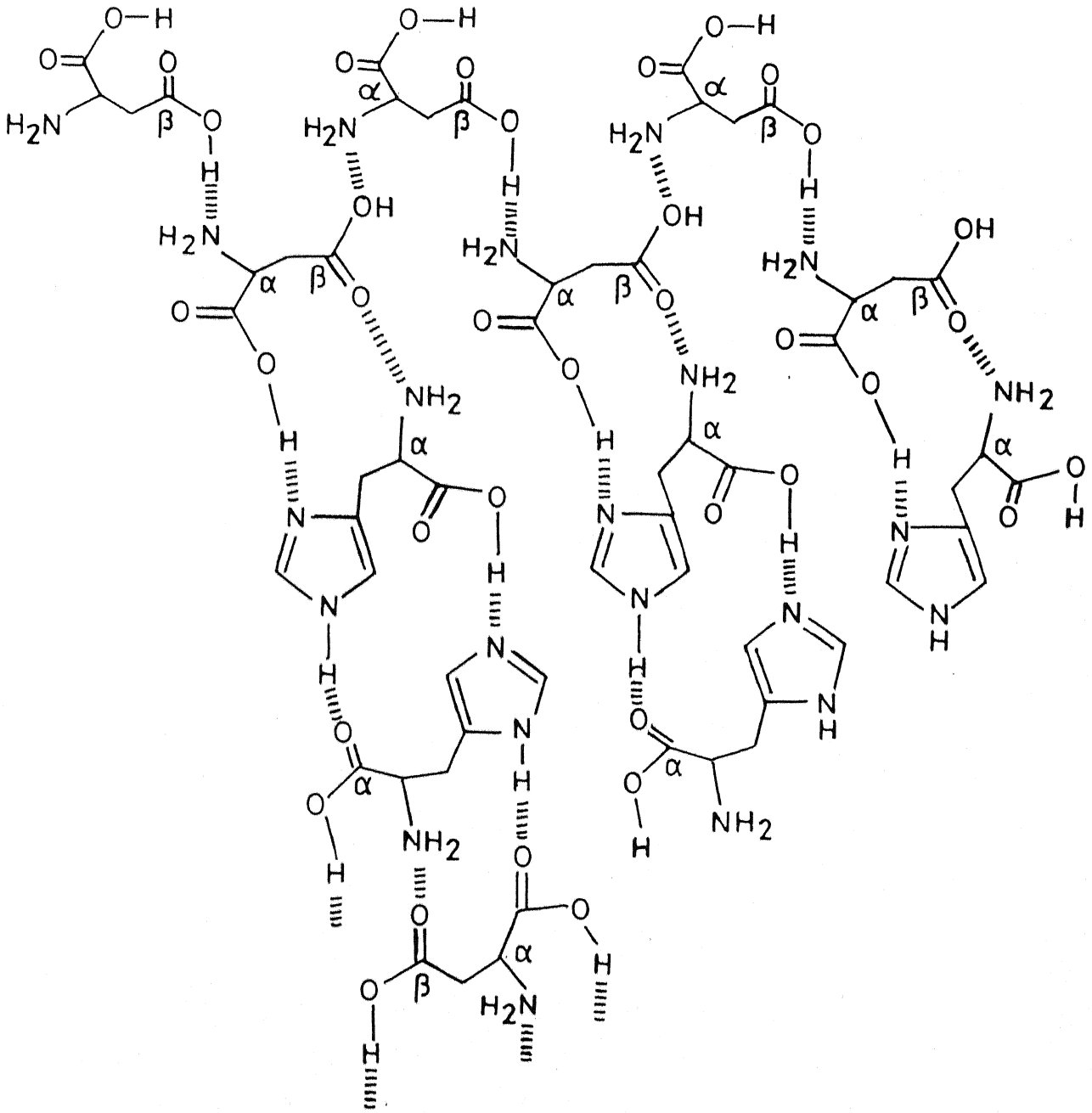
The diversity that exists in the side chains of the 20 coded amino acids, would most certainly, lead to preferences in alignments and the choice of proximate partners. Peptide bond formation taking advantages of such preferences, would lead to preferred peptide sequences that may well be precursors to present day enzymes. The work described in the present section outlines efforts directed at selective peptide bond formation based on molecular order and ranging from the most organized crystal lattice to the synthetically created micellar systems.

STUDIES ON SELECTIVE PEPTIDE BOND FORMATION IN A CRYSTAL LATTICE

Studies on the X-ray structures of pairs of amino acids, so chosen, that the side chains on mutual interaction , would lead to a salt showed most interestingly, ordered arrangements , without exception . The layers of amino acid pairs, were homogeneous. Of particular relevance to the present work is the crystal structure of aspartic acid - histidine, which is presented in CHART C.1.

The aspartic acid - histidine mixed crystals exhibit a highly ordered arrangement consisting of alternating bilayers of aspartic acid and histidine, wherein all the hydrogen bonding possibilities are taken advantage of . As could be seen from CHART C.1, the horizontal arrangement of aspartic acid residues is such that the β -COOH a l w a y s face the α -amino acid moiety of the neighbour. With reference to the histidine bilayers such a situation does not exist. In this case, the α -amino acid moiety faces the adjacent Asp β CO_2H

CHART C-1



ORDER IN ASP-HIS MIXED CRYSTAL

residue. An extraordinary feature of the arrangement illustrated in CHART C.1, is that the aspartic acid residue, either horizontally or with the adjacent layer, is lined up for the peptide bond formation in such a way that the β -peptides are formed.

The various possibilities for the peptide bond formation dictated by order such as illustrated in CHART C.1, is shown in CHART C.2.

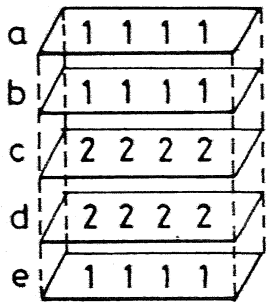
In CHART C.2, the repeating layers are designated as a,b,c and d. Peptide bond formation either involving a or b or a combination of both would give rise to NH_2-Asp(α -OH)-Asp(α -OH) - - - . On the other hand the peptide bond formation initiated by layer b can lead to the sequence 1 - 2- - 1-2- leading to NH_2Asp(α -OH)-His-Asp(α -OH)- - - - - - or 1 - 2 - 1 - 1- - leading to NH_2-Asp(α -OH)-His-Asp(α -OH)Asp(α -OH)-Asp(α -OH)-His - - - -. It was felt that the demonstration of the peptide bond formation in a mixed crystal such as presented in CHART C.1, without disturbing the crystal order would be of significance.

It was envisaged that this could be attained by co-crystallizing aspartic acid, histidine and a carbodiimide precursor, the latter so designed as to generate the condensing agent on photolysis, a process that most probably would do minimum damage to the crystalline arrangement.

A careful examination of the literature showed that the only molecular system that fulfills the above stated, rather stringent requirement is 1,4-dimethyl-tetrazoline -5-thione (1)[35]. This compound is reported to undergo photolytic double extrusion of N_2 and S leading to dimethyl carbodiimide. In the present work, it was considered appropriate to extend this to related models, namely, 1,4-dimethyl-5-diazo-tetrazoline (7) and the tetra amino ethylene (9) (CHART C.3).

CHART C-2

CRYSTAL ARRANGEMENT

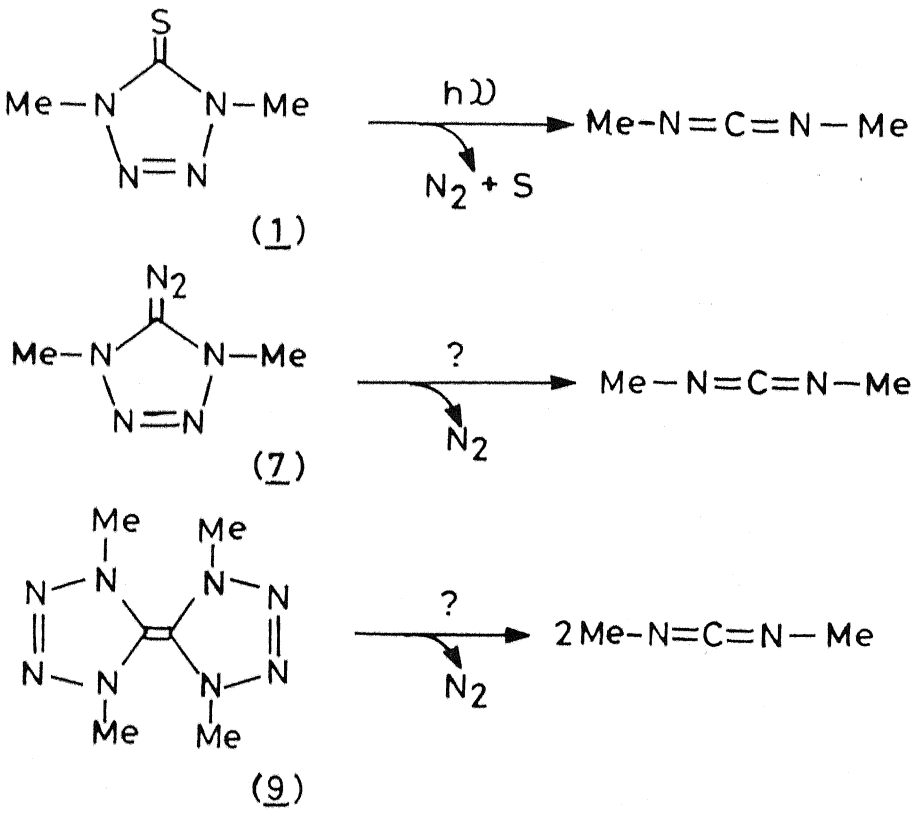


Layer a/b = 1 (Asp)
c/d = 2 (His)
e = 1 (Asp)

PEPTIDE POSSIBILITIES

LAYERS INVOLVED	SEQUENCE	PRIMARY STRUCTURE
a & a+b	1-1-1-1.....	H_2NAsp(α-OH)-Asp(α-OH) -----OH
b & c	1-2-1-2....	H_2NAsp(α-OH)-His-Asp(α-OH)----OH
b & c & a	1-2-1-1....	H_2NAsp(α-OH)-His-Asp(α-OH)- Asp(α-OH)-Asp(α-OH)-His ------

CHART C-3


S
Me–N
N–Me
N=N
hν
Me–N=C=N–Me
N2 + S
(1)
N2
Me–N
N–Me
N=N
?
Me–N=C=N–Me
N2
(7)
Me
Me
N
N
N
N
N
N
N
N
Me
Me
?
2 Me–N=C=N–Me
N2
(9)

The double extrusion of N_2 from (7) leading to the active condensing agent dimethyl carbodiimide was considered to take place with greater facility than with (1) involving extrusion of S and N_2. Compound (9) represents an interesting system wherein, the central π bond is liganded to nitrogen carrying lone pairs. Such systems are inherently unstable and could be used to form carbenic intermediates.[36] In the case of (9), such a carbenic intermediate can be anticipated to extrude N_2 readily with the formation of dimethyl carbodiimide.

The first phase of the work reported in this thesis thus envisaged the preparation of (1), (7) and (9), or related compounds, the use of such carbodiimide precursors to form crystals in conjunction with an amino acid pair whose structure is known - such as the case of Asp-His (CHART C.1), the determination of X-ray structures of the composite, the generation of dimethyl carbodiimide by photolysis and characterization of the nature of the peptide bond formed.

The preparation of 1,4-dimethyl-tetrazoline-5-thione (1) is illustrated in CHART C.4. The reaction of freshly prepared MeNCS with azide ion, followed by treatment with dimethyl sulfate gave 1-methyl-5-(methylthio)-1H-tetrazole (2). Compound (2), in turn, on neat heating with dimethyl sulfate led to the key methosulfate (3). The *in situ* demethylation of (3) gave the expected thione (1) in 45% yields

---

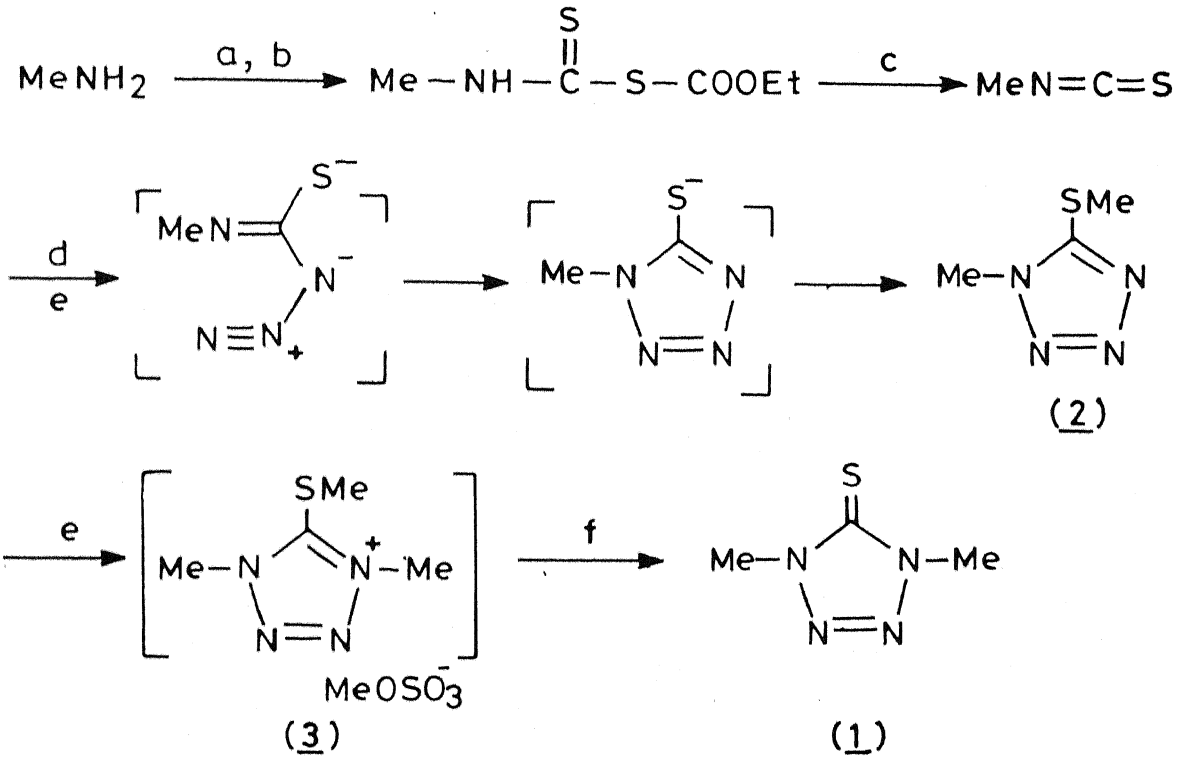
1,4-dimethyl-tetrazoline-5-thione (1) :

mp. 102-103°C

ir : ν_{max}(KBr) cm^{-1} : 1360 (C=S).

nmr : $\delta(CDCl_3)$: 3.9 (s, 6H, $(N\underline{Me})_2$).

CHART C-4



a : CS_2 , KOH ; b: ClCOOEt ; c : Δ ; d: Aq NaN_3 ;

e : $(MeO)_2SO_2$; f : NEt_3

ms : m/z : 130 (M^+), 73 $(MeNCS)^+$.

1-Methyl-5-(methylthio)-1H-tetrazole (2) :

bp. 138-140°/10 torr.

nmr : δ (CCl_4) : 2.8 (s, 3H, SCH_3), 3.9 (s, 3H, NCH_3).

ms : m/z : 130 (M^+).

The methosulfate intermediate (3) was considered as an appropriate starting material for diazo compound (7). The reaction of (3) with tosylhydrazide led to the formation of the expected tosylhydrazone (4). However, pyrolysis of Na salt of (4), under conditions normally employed for the transformation of tosylhydrazones to diazo compounds[37], did not succeed.

1,4-dimethyl-tetrazoline-5-tosylhydrazone (4) :

mp. 204-205°C.

ir : ν_{max}(KBr) cm^{-1} : 3250 (NH), 1620 (C=N), 1330, 1160 (SO_2).

nmr : δ ($CDCl_3$) : 2.5 (s, 3H, CH_3).

ms : m/z : 282 (M^+), 197 ($TsNHN\equiv CH^+$), 155 $(TsH^{\cdot})^+$.

Thermolysis of (4) either neat or in xylene resulted in the fragmentation of the molecule as evident by isolation of Ts containing product. The fate

of the other fragments could not be determined.

As an alternate route to (7), the hydrazone (6) was prepared by two different procedures. The reaction of *in situ* generated methosulfate (3) with benzhydrazide led to the benzhydrazone (5a) in 46% yields. In a similar manner, ethylcarbazate afforded the carbmethoxy hydrazone (5b) in 20% yields.

1,4-dimethyl-tetrazoline bezoylhydrazone (5a) :

mp. 221-222°C.

ir : ν_{max}(KBr) cm^{-1} : 3240 (NH), 1645, 1545 (amide).

nmr : δ ($CDCl_3$+ DMSO-d_6) : 3.69 (s, 6H, CH_3), 7.39 - 7.91 (m, 5H, aromatic).

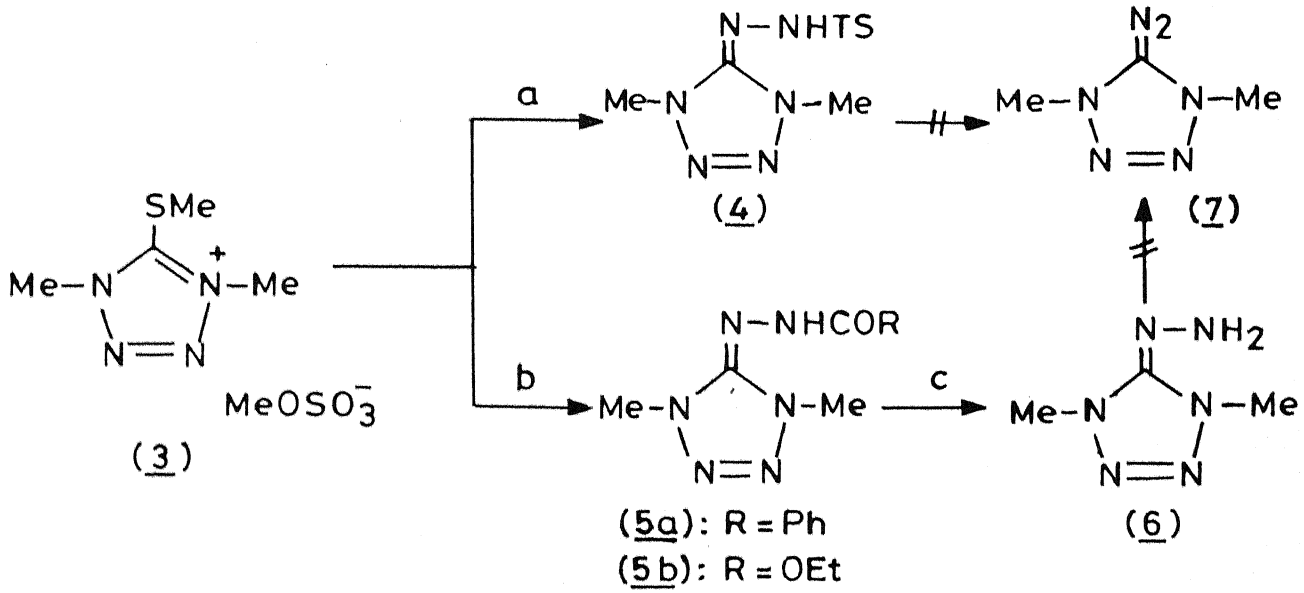
1,4-dimethyl-tetrazoline-5-ethyl carbazone (5b) :

mp. 120-124°C

ir : ν_{max}(KBr) cm^{-1} : 3480 (NH), 1720, 1625 (urethane).

Acidic hydrolysis of (5a) and basic hydrolysis of (5b), afforded the hydrazone (6) in poor yields. Attempted oxidation of this with HgO gave intractable mixtures (CHART C.5). Similarly, the attempted preparation of dimer (9), either from coupling of (1), mediated by trialkylphosphite or *via* union of the conjugate base of the tosylhydrazone (4) with (1) (CHART C.6), did not succeed. More determined persuits to diazo compound (7) and the dimer (9) were abandoned becaused of difficulties in the formation of mixed crystals involving 3 components (vide infra).

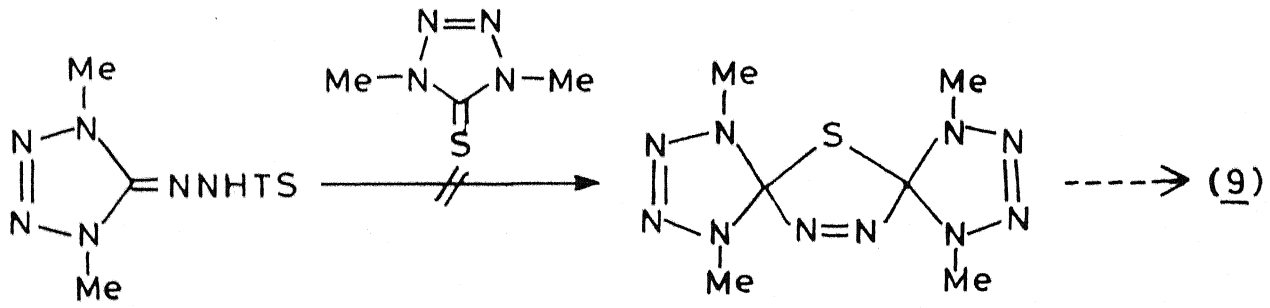
CHART C-5



a: $TSNHNH_2$, Py, Et_3N b: $BzNHNH_2$ / $EtOCONH_2$, Py, Et_3N

c: 6N HCl / 1N NaOH

CHART C-6

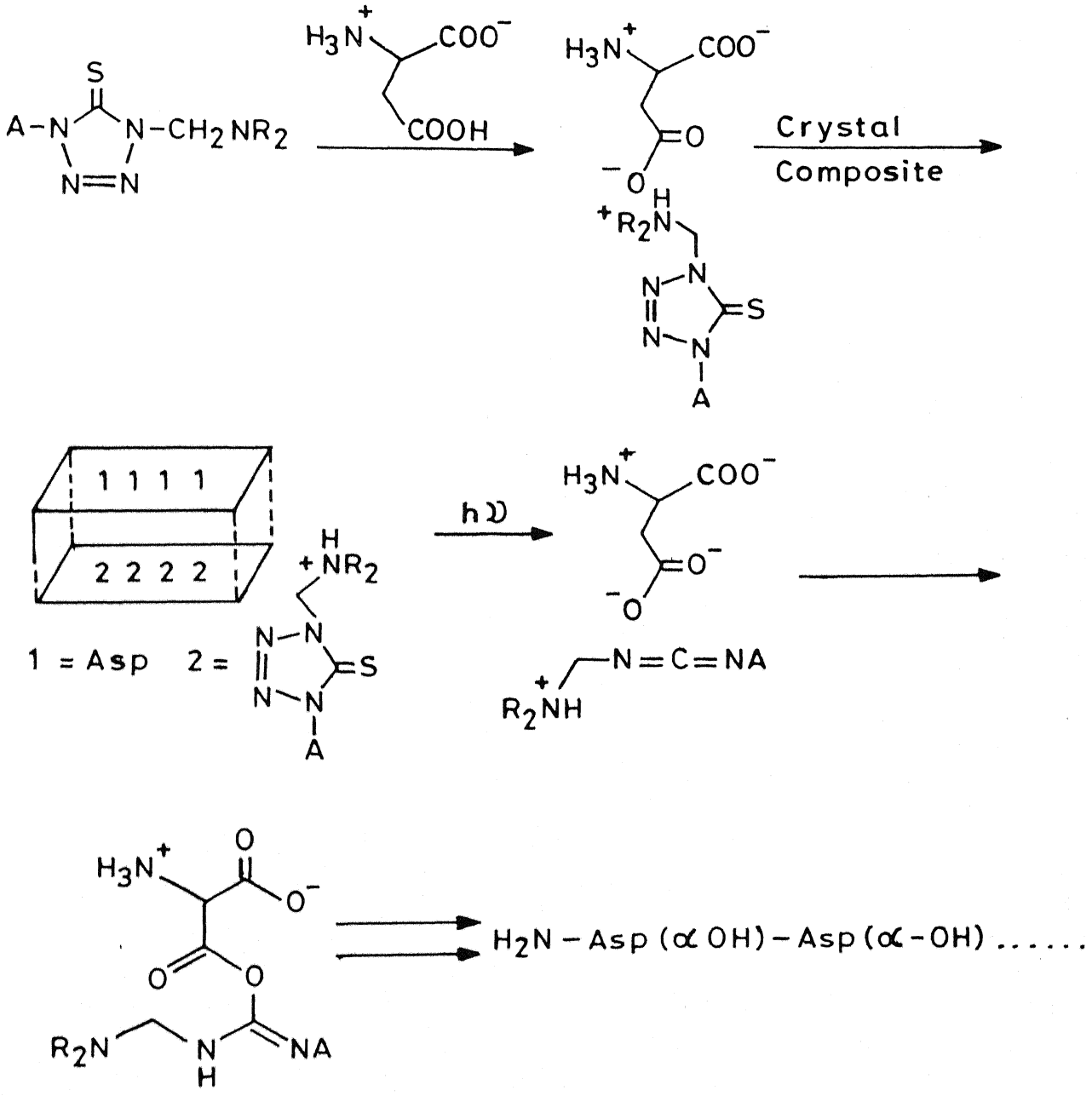


(4) (8)

Whilst, endeavours towards (7) and (9) were in progress, several experiments involving diverse techniques were performed to form mixed crystals, involving appropriate amino acid pairs and the thione (1). Extensive studies were done specifically with the objective of preparing aspartic acid + histidine + thione (1) mixed crystals. In most cases either from water or from aqueous acetone, the thione (1) crystallized out separately. The difficulties encountered could largely be due to problems associated with growing crystals incorporating multicomponents. Yet another difficulty could arise because of lack of interaction of the thione (1) with the amino acid systems studied. In order to reduce the number of components in growing crystal composites and in order to provide possibilities for salt formation, endeavours were made to attach either basic or acidic side chains to the operating part of thione (1). Thus, the replacement of one of the methyl ligands in (1) with either a basic or an acidic moiety would enable salt formation with the side chains of the appropriate amino acid, which, in turn, should facilitate the formation of crystal composite. Such crystals would retain the property to form the carbodiimide condensing reagent on photolysis which could lead to peptide bond formation in a crystal lattice. This approach is illustrated in CHART C.7 with Asp-Mannich base carbodiimide precursor.

1-Methyl-2-tetrazoline-5-thione (10) and 1-Phenyl-2-tetrazoline-5-thione (11) were considered as logical starting materials for attachment of appropriate side chains. In these compounds, the 4-nitrogen as well as the thione S of (10) could be the site for possible ligand attachment. However, it was felt that the site of alkylation can be controlled on the basis of reaction conditions and the nature of the product established by physical methods. Compounds (10) and (11) were prepared in, respectively, 46% and 84% yields by reaction of MeNCS/PhNCS with aqueous sodium azide (CHART C.8, CHART C.9).

CHART C-7


Crystal
Composite
1 1 1 1
2 2 2 2
1 = Asp 2 =
hν
$H_2N-Asp(\alpha OH)-Asp(\alpha-OH)$ .....

1-Methyl- 2 -tetrazoline-5-thione (10) :

mp. 125-127°C.

ir : ν_{max}(KBr) cm^{-1} : 1330 (C=S).

nmr : δ ($CDCl_3$) : 4.0 (s, 3H, CH_3), 7.4 (br, 1H, NH).

ms : m/z : 116 (M^+), 73 ($MeNCS^+$).

1-Phenyl- 2 -tetrazoline-5-thione (11) :

mp. 154°C.

ir : ν_{max}(KBr) cm^{-1} : 3500 (br, NH), 1360 (C=S).

nmr : δ ($CDCl_3$) : 7.6 (d, t, 3H, o,p.protons), 8.05 (m, 2H, m-protons).

It was thought that Mannich reaction would provide the easiest procedure for the preparation of tetrazole thiones, carrying a basic moiety. Indeed, the preparation of several such Mannich bases reported, although, the physical data pertaining to these were not available[38] . Surprisingly the simplest of the Mannich bases, namely, 1-methyl-4-dimethyl amino methyl tetrazoline-5-thione and 1-methyl-4- diethyl amino methyl-tetrazoline-5-thione could not be prepared by reaction of (10) with respectively, dimethylamine and diethylamine and formalin. In view of the fact that the diethyl amino methyl Mannich base has been claimed via a reaction of (11) with diethylamine and formalin[38] , our failure with (10) was surprising. In the event, however, repetition of the reaction of (11) with diethylamine and formalin gave compound having melting point

as reported but whose nmr clearly showed that this compound was not the Mannich base, but, to which, on the basis of physical data, the structure 1-phenyl-5-diethylamino tetrazoline (14) has been assigned, arising from nucleophilic attack on thione (11) followed by loss of H_2S. The yield of (14) was 58% (CHART C.9). A similar pathway would account for failure to obtain Mannich base from (10) with dimethylamine and diethylamine and formalin.

1-Phenyl-5-(diethylamino tetrazoline)(14) :

mp. 111°C.

nmr : δ ($CDCl_3$) : 1.5 (t, 6H, CH_2-($\underline{CH_3}$)$_2$), 3.15 (q, 4H, N($\underline{CH_2}$)$_2$-CH_3), 7.5 (d, t, 3H, o ,p.protons), 8.0 (m, 2H, m-protons).

Interestingly, piperidine and morpholine, gave Mannich bases easily. Thus, the reaction of (10) with piperidine and formalin gave rise to the expected 1-methyl-4-piperidinomethyl-tetrazoline-5-thione (13) in 56% yields. The structural assignments for (13) is supported by spectral and analytical data. The N-substitution is particularly evident from the nmr spectrum which exhibits a singlet at δ 5.2, clearly attributable to N-CH_2-N protons.

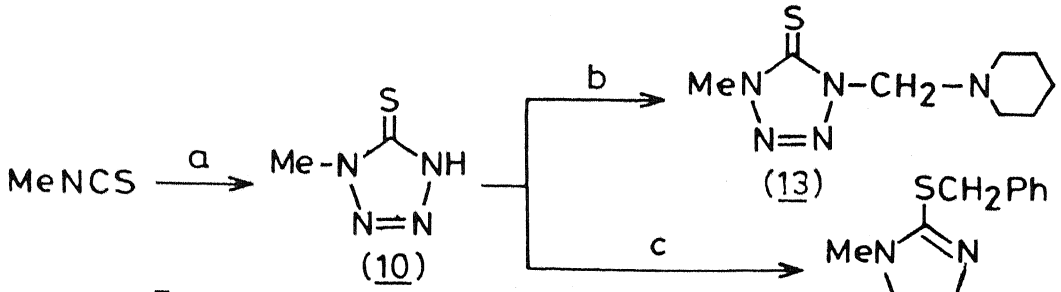
1-Methyl-4-(piperidinomethyl)-tetrazoline-5-thione (13) :

Oil

ir : ν_{max}(KBr) cm^{-1} : 1340 (C=S).

nmr : δ ($CDCl_3$) : 1.65 (m, 6H ($\underline{CH_2}$)$_3$), 2.7 (m, 4H, N-($\underline{CH_2}$)$_2$), 3.9 (s, 3H, N -$\underline{CH_3}$), 5.2 (s, 2H, N -$\underline{CH_2}$-N).

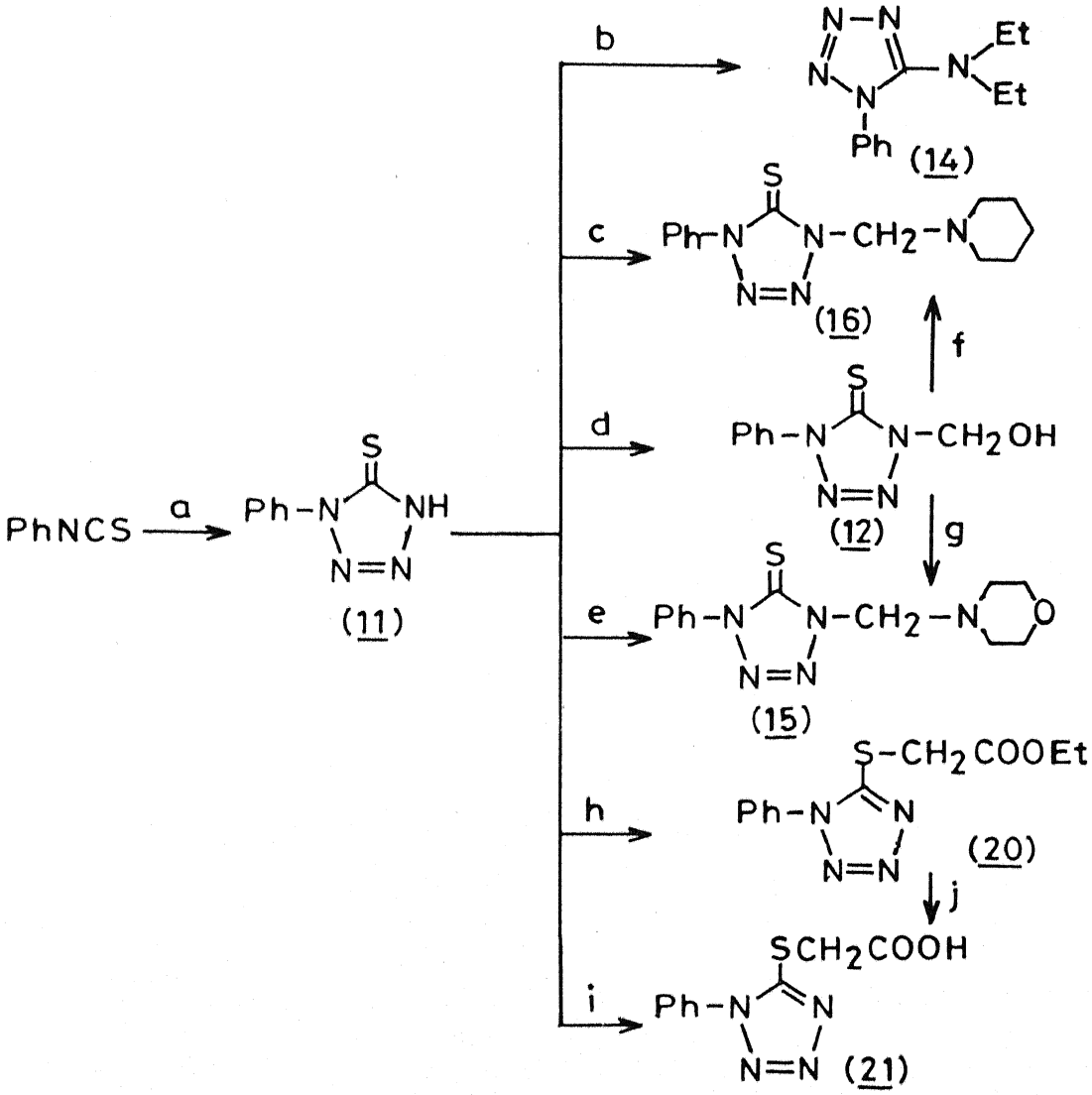
CHART C-8



a : aq N_3^-, reflux, b : formalin /pip/MeOH

c : $PhCH_2Br$, Et_3N

CHART C-9



a : aq N_3^-, reflux; b : formalin / Et_2NH / MeOH ; c : Formalin/pip ;

d : formalin ; e : formalin / morph ; f : pip ; g : morph ;

h : $BrCH_2CO_2Et$ / Et_3N / PhH ; i : $BrCH_2COOH$ / Et_3N / PhH ; j : INKOH

ms : m/z : 214 (M^++H), 154 ($MeN{=}C{=}N{-}CH_2{-}NHC_6H_{11})^+$, 101 ($MeNCSN{=}CH_2)^+$.

As stated earlier the choice of these, such as (1) was because of their being precursors to carbodiimides involving extrusion of S and N_2, although the mass spectrum of (1) itself did not exhibit such a fragmentation pattern (vide supra). The Mannich base (13) exhibited a prominent mass peak at m/z (154) which is attributed to the loss of N_2 and S from this compound. Thus, it appears that compounds such as (13) could lead to carbodiimides on thermolysis. Crystalline Mannich bases were obtained from 1-phenyl - 2 -tetrazoline-5-thione (11). Thus, the reaction of (11) and formalin with either piperidine or morpholine gave the expected Mannich bases (15) and (16) in respectively (67%), (74%) yields.

1-Phenyl-4-(morpholinomethyl)-tetrazoline-5-thione (15) :

mp. 154°C.

ir : ν_{max}(KBr) cm^{-1} : 1350 (C=S).

nmr : δ ($CDCl_3$) : 2.8 (t, 4H, $N(C\underline{H}_2)_2$), 3.7 (t, 4H, $O{-}(C\underline{H}_2)_2$), 5.3 (s, 2H, $N{-}C\underline{H}_2{-}N$), 7.5 (d, t, 3H, o,p,protons), 7.9 (m, 2H, m-protons).

1-Phenyl-4-(piperidinomethyl)-tetrazoline-5-thione (16) :

mp. 136-137°C.

ir : ν_{max}(KBr) cm^{-1} : 1350 (C=S).

nmr : ($CDCl_3$) : 1.5 (m, 6H, $(C\underline{H}_2)_3$), 2.3 (m, 4H, N-$(C\underline{H}_2)_2$), 5.3 (s, 2H, N-$C\underline{H}_2$-N), 7.5 (d, t, 3H, o,p,protons), 8.0 (m, 2H, m-protons).

Since the formation of (15) and (16) must involve a common N-CH_2-OH precursor, it was considered appropriate to prepare this compound. The reaction of (11) with formalin easily resulted in the formation of 1-phenyl-4(hydroxymethyl)-tetrazoline-2-thione-5 (12) (CHART C.9).

1-Phenyl-4(hydroxymethyl)-tetrazoline-5-thione (12) :

mp. 94°C.

ir : ν_{max}(KBr) cm^{-1} : 3300 (OH), 1360 (C=S).

nmr : $\delta(CDCl_3)$: 4.9 (OH), 5.8 (s, 2H, CH_2), 7.5 (d, t, 3H, o, p,protons), 8.0 (q, 2H, m-protons).

Parenthetically, compound (12) could, by loss of elements of water, lead to a protonated Schiff base intermediate that would be proximate to a thione grouping. Therefore, compound (12) could be used as a substrate for nucleophilic reactions as well as in cycloadditions. As expected, (12) on treatment with either piperidine or morpholine gave the Mannich bases (15) and (16). The route to this compound via (12) was found to be more practical (CHART C.9).

The case of transformation of hydroxymethyl compound (12) to Mannich bases (15) and (16) made it logical to use this compound for the preparation of the diethylamino compound which could not be obtained directly (vide supra).

In the event, the reaction of (12) with diethylamine without solvents resulted in the formation of a compound which was neither the expected Mannich base nor compound (14).

Having a range of Mannich bases at hand, endeavours were made to demonstrate the salt formation between the more readily available Mannich base (16) and carboxylic acid containing substrates. Clear filtrates resulting from aspartic acid and (16) were evaporated. The resulting solid melted over a range, its ir was not in agreement with that expected for the salt and the tlc, using Phenol - H_2O as developer followed by ninhydrin spray, clearly showed the presence of free aspartic acid. In view of the unsatisfactory results, it was considered necessary to demonstrate the salt formation between the Mannich base (16) and a more easily characterizable carboxylic acid containing substrate. Equimolar amounts of (16) and phenylacetic acid were finely ground and crystallized from 95% EtOH. The ir, tlc and melting point behaviour showed the absence of crystals of the salt. Similar results were obtained from methylene chloride solution of the piperidine Mannich base (16) and the morpholine Mannich base (15) and the phenylacetic acid. In all the above cases, although salt resulted, they could not be induced to provide crystals suitable for further investigations.

An alternate approach envisaged the attachment of an acetic acid chain to the N-4 position of the thione (11), this was endeavoured *via* treatment of (11) with ethylbromoacetate in benzene in presence of triethylamine. The crystalline compound thus obtained has been assigned the S-alkylated structure (20) on the basis of spectral data [39, 40](CHART C.9).

The N and S alkylated products arising from (10) and (11) can be easily distinguished on the basis of nmr. Thus 1-Benzyl - 2 - tetrazoline-5-thione (18) -prepared from benzyl-NCS and NaN_3 - on treatment with benzylbromide in

benzene in presence of NEt_3 gave 1-benzyl-5-(benzylthio)-1H-tetrazole (19) (CHART C.10). The nmr spectrum of compound (19) clearly distinguishes the N-alkylated and S-alkylated ligands, wherein the methylene protons appear, respectively, at δ 5.3 for N-$\underline{CH_2}$-Ph - similar to that observed in Mannich bases and at δ 4.5 for the S-$\underline{CH_2}$Ph. The latter assignment is confirmed via preparation of 1-Methyl-5-(benzylthio)-1H-tetrazole (17) - by reaction of (10) with C_6H_5-CH_2Br/NEt_3. In compound (17) the S-$\underline{CH_2}$-Ph appeared at 4.5 ppm as in the case of (19). The fact that the methylene protons arising from alkylation of (11) with $BrCH_2CO_2Et$ appeared at δ 4.2 clearly indicates S-alkylation. The reaction of (11) with $BrCH_2CO_2H/NEt_3$ again resulted in S-alkylation leading to (21). Compound (21) could also be prepared by saponification of (20). An unusual feature of (21) is the appearance of the methylene protons as a doublet. Whilst the intensities of the two peaks of the product arising from the saponification of (20) were nearly equal, that obtained from direct alkylation exhibited the two peaks of different intensities. In addition, the ir spectrum of this compound clearly showed salt like characteristics. Consequently, the product is considered as a mixture of the expected acid and the corresponding zwitter-ionic product.

1-Phenyl-5-(thioacetate)-1H-tetrazole (20) :

mp. 84-6°C.

ir : ν_{max}(KBr) cm^{-1} : 1740 (ester).

nmr : δ ($CDCl_3$) : 1.3 (t, 3H, O-CH_2-$\underline{CH_3}$), 4.25 (s, 2H, S-$\underline{CH_2}$), 4.3 (q, 2H, O-$\underline{CH_2}CH_3$), 7.6 (s, 5H, aromatic).

1-Phenyl-5-(thioacetic acid)-1H-tetrazole (21) :

Oil

ir : ν_{max}(neat) cm^{-1} : 3700 - 2300 (br), 1710 (COOH).

nmr : δ ($CDCl_3$) : 4.2 - 4.3 (2H, $C\underline{H}_2$,-COOH), 7.32 - 8.2 (m, 5H, aromatic).

ms : m/z : 237 MH^+

This finding is similar to results obtained on treatment of (18) with alkyl halides [40].

1-Benzyl-2-tetrazoline-5-thione (18) :

mp. 143°C.

ir : ν_{max}((KBr) cm^{-1} : 3090 (NH), 1360 (C=S).

nmr : δ ($CDCl_3$) : 5.4 (s, 2H, $NC\underline{H}_2$-Ph), 7.4 (br, 5H, Ph).

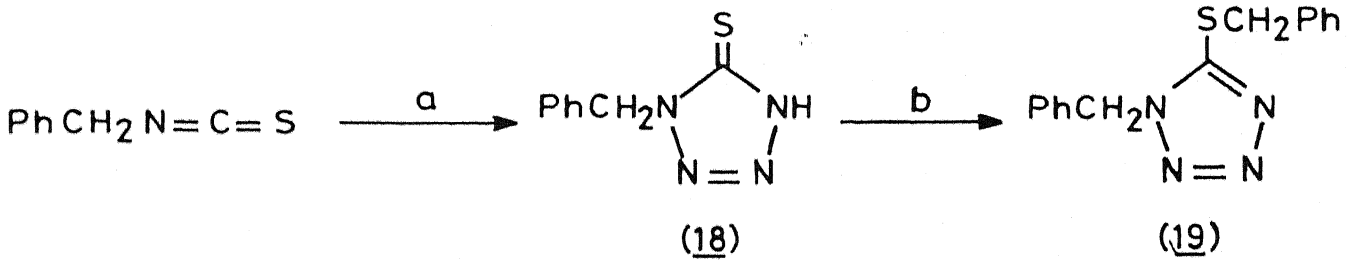
1-Benzyl-5-(benzylthio)-1H-tetrazole (19) :

mp. 59-60°C.

ir : ν_{max}(KBr) cm^{-1} : 3000 (-NH), 1610 (C=N), 1505 (N=N).

nmr : δ ($CDCl_3$) : 4.5 (s, 2H, S-$C\underline{H}_2$-Ph), 5.3 (s, 2H, $NC\underline{H}_2$-Ph), 7.3 (br, 10H, aromatic).

CHART C-10



(18) (19)

a : aq N_3^- , b : $PhCH_2Cl$, NEt_3 , Et_2O .

1-Methyl-5-(benzylthio)-1H-tetrazole (17) :

Oil

nmr : δ ($CDCl_3$) : 3.75 (s, 3H, N-CH_3), 4.5 (s, 2H, S-CH_2), 7.3 (s, 5H, aromatic).

ms : m/z : 206 (M^+).

The N versus S alkylation of system represented (10) and (11) can be rationalized on the basis of HSAB principle. On the basis of this, the attachment of a propionic acid side chain to (11) was attempted *via* addition of acrylate. These endeavours did not succeed [40].

The experience arising from the work described above clearly brought out two major problems, namely, the preparation of tailor made carbodiimide precursors and the preparation of crystal composites. Whilst the synthesis of a range of Mannich bases demonstrate that the former objective can be achieved, the uniform failure in endeavours to produce crystal composites does tend to show that this problem could be very severe. Therefore, alternate methodologies for the demonstration of selective peptide bond formation was explored and these are described below.

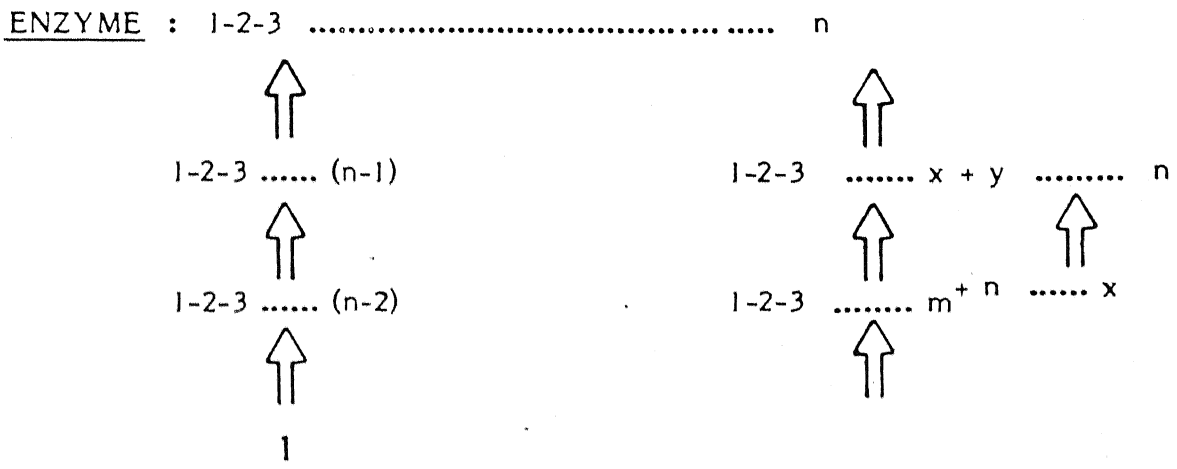
EXPERIMENTAL AND THEORETICAL APPROACH TO SELECTIVE PEPTIDE BOND FORMATION IN AQUEOUS MEDIA :

The order that exists in crystal lattice harboring a pair of amino acids, could logically be extended to their concentrated aqueous solutions. In this event, the condensation of amino acids, taking advantage of this order, can be achieved directly using water soluble carbodiimides. Interestingly, such a selective peptide bond formation in a milieu is also related to the problem of peptide bond formation in the very early stage of protein evolution. A question that has been raised for a number of years, is the possible preferences in the formation of peptide bonds and peptide sequences. In terms of strategies currently understood in organic syntheses, the establishment of such preferences would have a direct bearing on not only the evolution of functional system but also the possible methodologies by which the enzymes were constituted. This aspect is illustrated in CHART C.11.

If one considers the synthesis of an enzyme having an amino acid sequence $1 \rightarrow n$, a retro synthetic analysis would offer two broad possibilities. Thus, polypeptide could be made either by the addition of a single unit or in a convergent approach by joining existing preferred sequences. The efficiency of the latter approach is significantly higher and consequently would be a more favoured pathway for the production of complex enzymes. This notion would lead to the logical poser as to what such preferred segments would be. As shown in CHART C.12 the preferences in peptide bond formation involving all the 20 coded amino acids in aqueous medium can be examined by two methodologies.

CHART C-11

EVOLUTION OF FUNCTIONAL SYSTEM



ADDITION BY SINGLE UNITS

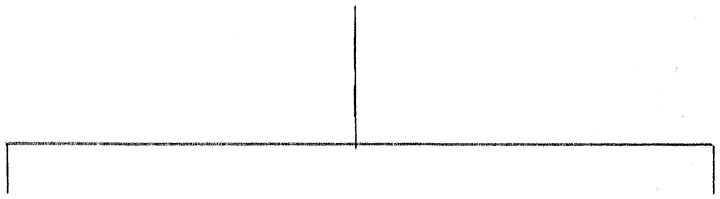
INEFFICIENT

ADDITION OF MULTI-UNITS (CONVERGENT APPROACH)
MORE EFFICIENT

THEREFORE THE FUNCTIONAL SYSTEM EVOLVED FROM COMBINATION OF EXISTING SMALL PEPTIDES

CHART C-12

WOULD THERE BE PREFERENCES IN PEPTIDE BOND FORMATION WHEN ALL THE 20 CODED AMINO ACIDS ARE PRESENT IN WATER IN PRESENCE OF A CONDENSING AGENT ? WOULD THERE BE A CHOICE OF NEIGHBOURS ?



EXPERIMENTAL APPROACH

OF THE n NUMBER OF POSSIBILITIES WHEN AMINO ACIDS a & b ARE MADE TO COMBINE, DETERMINE THE RATIO OF PEPTIDES FORMED.

THEORETICAL APPROACH

A COMPUTER BASED ANALYSIS OF A SIGNIFICANT NUMBER OF ESTABLISHED PROTEIN SEQUENCES SHOULD PROVIDE EVIDENCE FOR PREFERRED POLYPEPTIDE UNITS AS SYNTHONS.

The experimental approach, as shown in CHART C.12, envisaged the combination of unprotected amino acids a and b in clear solutions in water using a water soluble condensing agent and delineation of preferences in dipeptide formation via analytical methods. In other words, such an experiment would show the preferences in dipeptide formation, amongst the number of possibilities, in terms of quantities of products formed. It could be readily seen that this methodology would require, even for a pair of amino acids, extensive experimentation. For example, Glutamic acid and Leucine could combine to form six dipeptides (vide infra). In order to establish preferences, if any, the product mixture has to be compared with each of the authentic dipeptide samples. Whilst, the demonstration of such selectivity would constitute a novel finding, it would represent only a pointer and a preliminary observation. In spite of this, it was felt that a beginning can be made which could be further developed as a methodology related to two components and multicomponent systems. Regardless of the relationship between such preferences and protein evolution, it was felt that the establishment of such preferences by experimentation would lead to a better understanding of the side chain interactions of the coded amino acids in water.

In the event preferences do exist with respect to the choice of neighbours and consequently a preferred sequence, the intriguing poser arises as to whether such selectivity can be discerned in present-day proteins and if so, what would be its significance pertaining to protein evolution.

A key turning point in evolution leading to life would be the error free replication of the information molecule. This could be achieved only via enzymes. Stating this in a different way, it is logical to assume that those enzymes which are capable of replicating the information system without error,

would be sustained and improved upon. This would be possible only on the basis of an existing relationship between primary nucleic acid sequences on the one hand and the primary amino acid sequences on the other. The establishment and evolution of this relationship should reflect in the choice of amino acid neighbours and hence the existence of preferred sequences[41].

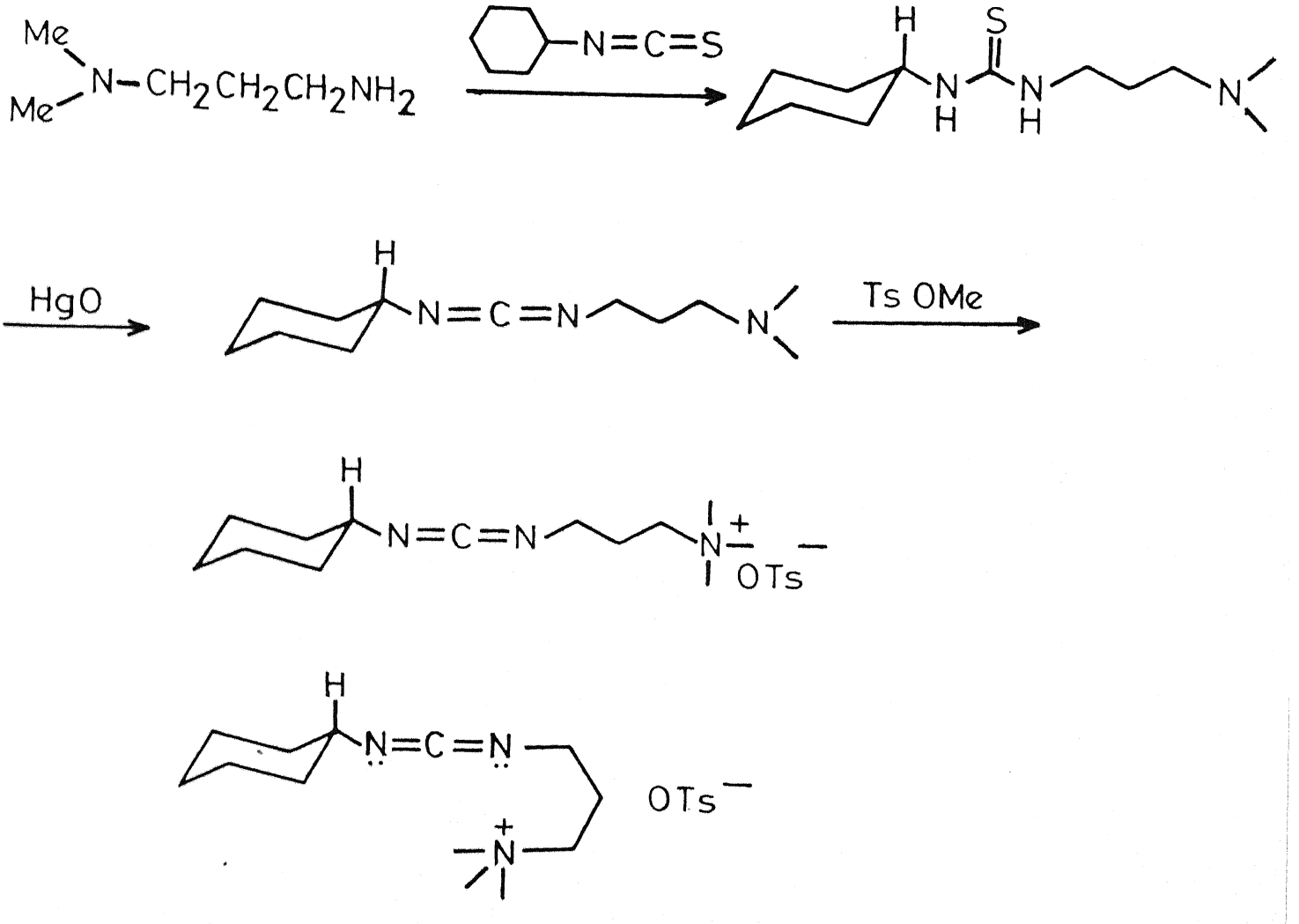
Most protein alterations in Nature can be traced through point mutation of the information system. The changes that can take place in the primary amino acid sequence as a function of point mutation can be easily known *via* relationship through the genetic code. An important reason is the finding that even amongst the numerous variations that can take place as a function of point mutation, some are clearly sustained over others[42]. It is possible that here also side chain preferences play a role.

In view of the above, it was considered to be of interest to compliment preferences established *via* experimentation with those present in biologically active proteins. The achievement of the latter objective would entail the development of a computer program that would analyse the frequency of the neighbours. This has been done in the present work (*vide infra.*). The overall objectives pertaining to the present sub-section are presented in CHART C.12.

The water soluble carbodiimides used in the present study are : 1-Cyclohexyl-3(3-dimethylaminopropyl)carbodiimide metho-p-toluene sulphonate (22) and 1-Cyclohexyl-3(3-diethylaminopropyl)carbodiimide etho-p-toluene sulphonate (25) (CHART C.13).

The reaction of N,N-dimethylaminopropylamine with cyclohexylisothiocyanate gave thiourea (23) which was converted to carbodiimide (24) by HgO oxidation. The terminal nitrogen was quaternized by transmethylation

CHART C-13


Me
N–CH2CH2CH2NH2
Me
N=C=S
H
S
N
H
N
H
N
HgO
H
N=C=N
N
Ts OMe
H
N=C=N
N
OTs
H
N=C=N
N
OTs

with methyl-p-toluene sulphonate yielding (22). The overall yield was 35%.

1-Cyclohexyl-3(3-dimethylaminopropyl)carbodiimide metho-p-toluene sulphonate (22) :
mp. 158°C.

ir : ν_{max}(KBr) cm^{-1} : 2140 (carbodiimide)

nmr : δ (D_2O): 1.4 (br, 12H, $(CH_2)_5$, N-CH_2-CH_2-CH_2-N), 2.3 (s, 3H, CH_3), 2.92 - 3.6 (br, 14H, $Me_3\overset{+}{N}$-CH_2-CH_2-CH_2-N + tert.proton), 7.4 (d,d, 4H, aromatic).

1-Cyclohexyl-3(3-dimethylaminopropyl)thiourea (23) :
mp. 71°C.

ir : ν_{max}(KBr) cm^{-1} : 3240 (-NH), 1650 (thiourea).

1-Cyclohexyl-3(3-dimethylaminopropyl)carbodiimide (24) :
Thick syrup

ir : ν_{max}(neat) cm^{-1} : 2160 (carbodiimide).

Compound (22) is freely soluble in water and is very efficient in the formation of peptide bonds in aqueous medium. Further, the carbodiimide (22) can be recovered unchanged by keeping in pH 10 borate buffer for 1 hour and rather surprisingly in water for 3 hours. The peptide bonds are formed with (22) using equimolar amounts of the substrates and the reagent. The fact that

that dipeptides are formed in very good yields clearly show that the reaction of (22) with the carboxyl function of the amino acid leading to an activated ester is overwhelmingly preferred over the reaction of (22) with water. This is an interesting observation particularly since the activated ester formation with (22) really mimics similar processes that take place in living systems. It is possible that, in water, (22) is folded in such a manner so as to maximize lone pair $^{+}$ charge interaction (as shown in CHART C.13),which would give a measure of protection to the carbodiimide from water.

1-Cyclohexyl-3(3-N,N-diethylaminopropyl)carbodiimide etho-p-toluene sulphonate (25) was prepared in a analogous manner from N,N-diethylaminopropylamine via thiourea (26) and the carbodiimide (27) in a overall yield of 34%.

1-Cyclohexyl-3(3-N,N-diethylaminopropyl)carbodiimide etho-p-toluenesulphonate (25) :

mp. 148-150°C.

ir : ν_{max}(KBr) cm^{-1} : 2145 (carbodiimide).

1-Cyclohexyl-3(3-N,N-diethylaminopropyl)thiourea (26) :

mp. 74°C.

ir : ν_{max}(KBr) cm^{-1} : 3210 (NH)

nmr : δ ($CDCl_3$) : 0.9 (t, 6H, 2 x CH_2-$\underline{CH_3}$), 2.5 (m, 6H, 3 x N -$\underline{CH_2}$), 3.5 (m, 3H, $\underline{CH_2}NH(CS)NH\underline{CH}$).

ms : m/z : 271 $(M+H)^+$, 199 (277-$(NHEt_2)$).

1-Cyclohexyl-3(3-N,N-diethylaminopropyl)carbodiimide (27) :

Thick syrup

ir : ν_{max}(neat) cm^{-1} : 2125 (carbodiimide).

nmr : $\delta(CDCl_3)$: 1.0 (t, 6H, 2 x $CH_2\underline{CH_3}$), 2.5 (m, 6H, 3 x N-$\underline{CH_2}$), 3.25 (t, 2H, $\underline{H_2}C$-N=), 3.5 (br, 1H, tert.proton).

ms : m/z : 183 (256 - Et_2NH), 256 ($MH^+ + H_2O$).

It was surprising, that, in view of the excellent potential of water soluble carbodiimides, they have found only very limited applications[13-25]. A particularly noteworthy recent application of these systems is in the production of antibody enzymes (abzymes)[43].

At the outset, the efficiency of (22) and (25) in bringing about the peptide bond formation was tested with model substrates. The reaction of (25) with N-phthaloyl glycine and glycine methyl ester in equivalent amounts and in aqueous medium for 6-8 hours resulted in the formation of the dipeptide N-phthaloyl Glycyl-Glycine methyl ester (28) in 83% yields. Parenthetically, N-phthaloyl Glycine has limited solubility in water. The importance of water solubility is clearly brought out in the reaction of N-phthaloyl Phe and leucine-methyl ester - both of which have limited solubility - with (25) in water for 4 hours. In this case the yields of the dipeptide (29) was considerably less (46%).

N-Phthaloyl-Gly-Gly-OMe (28) :

mp. 194°C.

ir : ν_{max}(KBr) cm^{-1} : 1780, 1650 (Phth), 1740 (ester).

nmr : δ($CDCl_3$): 3.7(s, 3H, $COOC\underline{H}_3$), 4.0 (d, 2H, $NH-C\underline{H}_2-COOCH_3$), 4.4 (s, 2H, $NC\underline{H}_2-COOMe$), 6.4 (br, 1H, NH), 7.7 (m, 4H, aromatic).

ms : m/z : 276 (M^+)

N-Phth-Phe-Leu-OMe (29) :

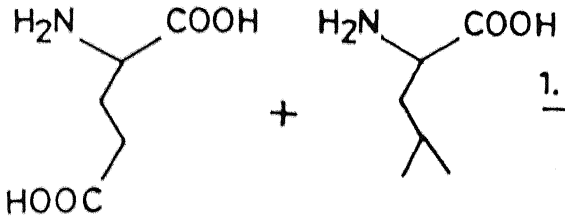
mp. 112-115°C.

ir : ν_{max}(KBr) cm^{-1} : 1780, 1660 (Phth), 1740 (ester).

nmr : δ($CDCl_3$) : 1.0 (d, 6H, $CH\underline{Me}_2$), 1.65 (m, 3H, $CH-C\underline{H}_2C\underline{H}Me_2$), 3.6 (d, 2H, $C\underline{H}_2-Ph$), 3.7 (s, 3H, $COOC\underline{H}_3$), 4.6 (m, 1H, $CONH-C\underline{H}$), 5.2 (t, 1H, $N-C\underline{H}-CH_2$), 6.7 (m, 1H, NH), 7.15 (s, 5H, Ph), 7.7 (s, 4H, Phth).

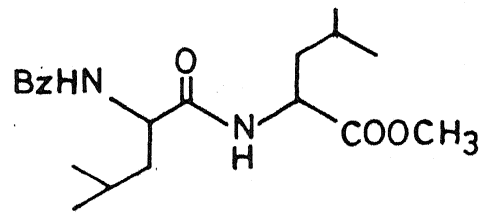
The first experiment with specific objective that was carried out with the water soluble carbodiimide (22) was the reaction of Glutamic acid with Leucine. The objective was to determine whether there would be preferences in peptide bond formation between Glu and Leu. Equivalent amounts of Glu, Leu and (22) in clear aqueous solution was left stirred for 2 days and the resulting dipeptide mixture was N,C-protected using BzCl-NaOH and CH_2N_2. As illustrated in CHART C.14, six N,C-protected dipeptides could arise, namely, Glu(γ -OH)-Leu, Glu(α -OH)Leu, Glu(α -OH)Glu, Glu(γ -OH)-Glu, Leu-Glu and Leu-Leu.

Since the aim of the experiment was to determine the composition of each of the above mentioned six possibilities, it was necessary, at the outset,

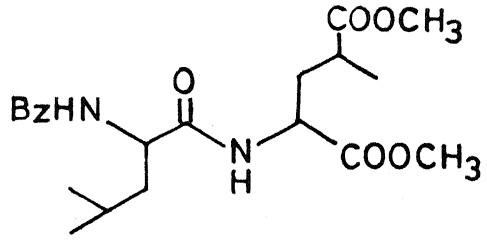


GLU (1) + LEU (1)

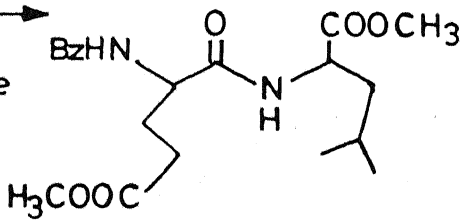
1. Clear solution in water + water soluble carbodiimide 2d, rt
2. BzCl-NaOH
3. CH_2N_2



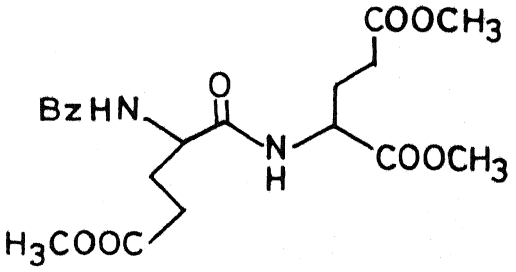
Bz LEU-LEU-OMe (30)



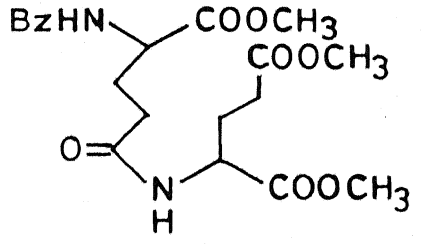
Bz LEU-GLU-DiOMe (31)



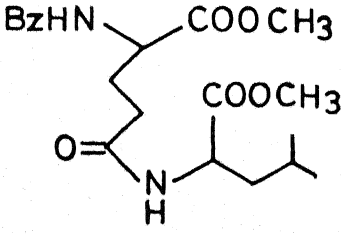
Bz GLU(γ-OMe)-LEU-OMe (32)



Bz GLU(γ-OMe)-GLU-DiOMe (33)



Bz GLU(α-OMe)-GLU-DiOMe (34)



Bz GLU(α-OMe)-LEU-OMe

to prepare authentic samples for comparison purposes.

Bzleu-leu-OMe (30), Bzleu-GludiOMe (31), BzGlu(γ -OMe)-LeuOMe (32), BzGlu(γ -OMe)-GludiOMe (33) were prepared in respectively, 39%, 48%, 45%, 68% by condensation of the appropriate partners using DCC-HOBt in methylene chloride at rt.

Bzleu-leu-OMe (30) :

mp. 200°C.

ir : ν_{max}(KBr) cm^{-1} : 3320, 3260 (-NH), 1740 (ester), 1620, 1520 (amide).

nmr : δ ($CDCl_3$) : 0.9 (d, d, 12H, 2 x CH-(CH_3)$_2$), 1.7 (m, 6H, 2 x CH_2CH Me_2), 3.75 (s, 3H, $COOCH_3$), 4.7 (m, 2H, tert.protons), 6.75 (br, 2H, NH), 7.6 (m, 5H, aromatic).

ms : m/z : 362 (M^+), 363 $(M+H)^+$, 306 (M^+-(isobutylene)), 218 (M^+-(NHCH(E)CH_2ipr)), 190 $(BzNHCHCH_2ipr)^+$.

Bzleu-GludiOMe (31) :

mp. 119-120°C.

ir : ν_{max}(KBr) cm^{-1} : 3250 (NH), 1735 (ester), 1625, 1525 (amide).

nmr : δ ($CDCl_3$) : 1.0 (d, 6H, CH-Me_2), 1.6 - 2.5 (m, 7H, methylenes, CH-Me_2), 3.58, 3.7 (s, s, 3H, α -COOMe), 3.6 (s, 3H, γ -$COOCH_3$), 4.0 - 5.0 (m, 2H, tert.protons), 6.7 - 7.9 (m, 7H, aromatic + 2xNH).

ms : m/z : 392 (M^+), 336 (M^++H-(isobutylene)), 218 (M^+-(NHCH(COOMe)CH_2CH_2COOMe), 190 $(BzNHCHCH_2ipr)^+$.

BzGlu(γ -OMe)-leuOMe (32) :

Thick syrup

ir : ν_{max}(neat) cm^{-1} : 3280 (-NH), 1730 (ester), 1520, 1640 (amide).

nmr : δ ($CDCl_3$) : 0.9 (d, 6H, $CH\underline{Me}_2$), 1.4 (m, 1H, $C\underline{H}$-Me_2), 1.6 (m, 2H, $C\underline{H}_2$-$CHMe_2$), 2.2 - 2.9 (m, 4H, $CHC\underline{H}_2C\underline{H}_2COOMe$), 3.65 (s, 3H, γ -$COOC\underline{H}_3$), 3.75 (s, 3H,α -$COOC\underline{H}_3$), 4.7 (m, 2H, tert.protons), 7.1 - 7.8 (m, 7H, 2 x N$\underline{H}$ + aromatic).

ms : m/z : 393 $(M+H)^+$, 248 (M^+-(NHCH(COOMe)CH_2ipr), 220 (BzNHCH $CH_2CH_2COOMe)^+$.

BzGlu(γ-OMe)-GludiOMe (33) :

mp. 119-120°C.

ir : ν_{max}(KBr) cm^{-1} : 3280, 3215 (-NH), 1740, 1730 (ester), 1620, 1520 (amide).

nmr : δ ($CDCl_3$) : 2.0 - 2.7 (m, 8H, methylenes), 3.6, 3.65 (s, s, 3H, 3H,2xγ-$COO\underline{Me}$), 3.7 (s, 3H,α- $COO\underline{Me}$), 4.7 (m, 2H, tert.protons), 7.3 - 7.9 (m, 5H, aromatic).

ms : m/z : 423 $(M+H)^+$, 220 $(BzNHCHCH_2CH_2COOCH_3)^+$, 174 (NHCH (COOMe)$CH_2CH_2COOMe$).

In Glutamic acid, the carboxyl groups are in different steric environments. Because of this, whilst, it is easy to prepare specifically the γ-ester, thus, enabling peptide bond formation involving the α-CO_2H, the reverse is very difficult. In the present work, the specifically α-protected Glutamic acid, namely, BzGlu(α-OMe)-OH, was prepared in a novel manner via oxidative hydrolysis of N-BzProOMe with Ru^{VIII} species leading to BzGlu(α-OMe)-OH and Bz-pyro-glu-OMe.

The former was used in the preparation of authentic BzGlu(α-OMe)-GludiOMe (34) in 54% yields.

BzGlu(α-OMe)-GludiOMe (34) :

Sticky solid

ir : ν_{max}(neat) cm^{-1} : 3290 (-NH), 1750 (ester), 1550, 1645 (amide).

nmr : δ ($CDCl_3$) : 1.9 - 2.7 (m, 8H, methylenes), 3.6 (s, 3H, γ-COOMe), 3.62 (s, 3H, α-COOMe), 3.7 (s, 3H, α-COOMe), 4.7 (m, 2H, tert. protons), 7.32 - 7.9 (m, 7H, aromatic + 2 x NH).

ms : 363 (M^+ -COOMe), 174 ($NH\text{-}CHCO_2Me\text{-}CH_2CH_2CO_2Me)^+$, 220 ($CH_2CH_2CHCOC_6H_5NHCO_2Me)^+$.

The authentic sample of the remaining dipeptide, namely, BzGlu(α-OMe)-leuOMe could not be prepared due to paucity of BzGlu(α-OMe)OH [44].

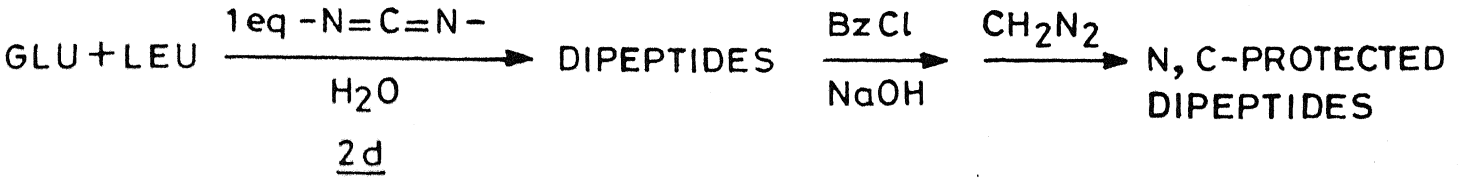
In the initial experiment, the crude dipeptide mixture was subjected to preparative tlc which resulted in the isolation of N-benzoyl-γ-Methyl-L-glu-

tamyl leucine methyl ester BzGlu(γ -OMe)leuOMe (32) in 32% yields. The structural assignment was confirmed by comparison with an authentic sample. This experiment showed that it would be very difficult to isolate the other products by preparative tlc. Therefore it was decided to use HPLC as the method for analysis of the dipeptide mixtures. In the event, the reaction was carried out and the dipeptide reaction mixture was N,C-protected as described above and subjected to HPLC analysis. The HPLC analysis of the reaction mixture and the reference samples were done on the same day to avoid minimization of column aberrations. The result obtained from the HPLC study was correlated with that from comparison of authentic samples and the products by nmr and is presented in TABLE C.1. It can be clearly seen from TABLE C.1 that regardless of finer aspects of reaction mechanisms, a clear preference for the formation of some dipeptides over others exist. This is the first time such a preference is demonstrated. Parenthetically, since the N,C-protection procedures used hardly affected the dipeptide structures present, the results presented in TABLE C.1 should also be related to the preference in the formation of the peptide bond between Glu and Leu.

TABLE C.1 represents the first ever experimental study pertaining to peptide bond formation amongst unprotected amino acids in water and exhibits several noteworthy features.

In the peptide bond formation involving Glutamic acid that takes place in every cell, the more hindered α -carboxyl group forms the activated ester. The fact that a similar trend is evident in the present experiment which mimics in vivo reaction, is perhaps the most satisfying result of this experiment. In other words the preference for peptide bond formation involving the α -carboxyl over γ -carboxyl grouping of Glutamic acid is, 72 : 15 TABLE C.1. Although,

TABLE C-1



HPLC ANALYSIS RESULTS

	(%)
Bz - GLU (γ - OMe) - LEU - OMe	72
Bz - GLU (α - OMe) - GLU - DiOMe	15
Bz - LEU - LEU - OMe	8
HIGHER PEPTIDES	1.5
	96.5

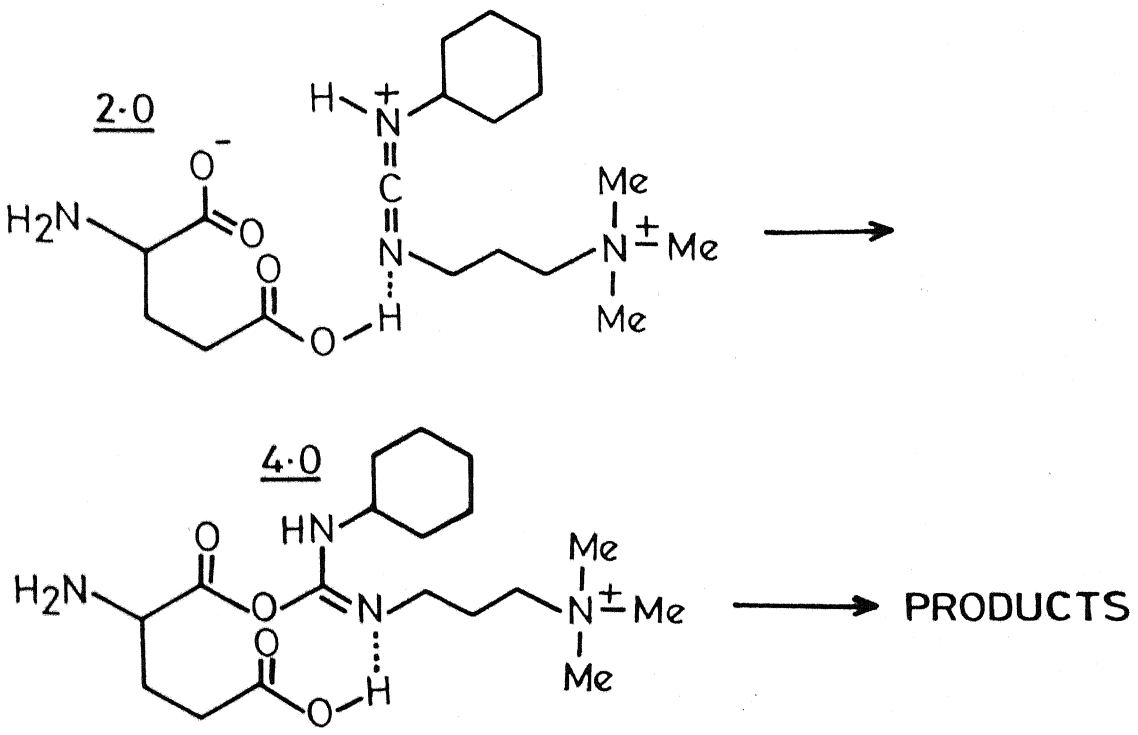
GLU + GLY ------→ HPLC SHOWS PREFERENTIAL GLYCINE POLYMERIZATION

solubility problems preclude a comparison of present results with similar ones in non-polar media, there is ample evidence to show that under these conditions the γ -carboxyl group is much more reactive to ester formation (vide-supra) . The specific activation of the sterically more encumbered α -CO_2H grouping can be understood on the basis of a preference in the salt formation between the more acidic α -carboxyl group and the water soluble carbodiimide (22). This is illustrated in CHART C.15. The pka of α - and γ -CO_2H groups of Glutamic acid are, respectively 2 and 4. It is envisaged in CHART C.15 that whilst the more acidic α -CO_2H group is involved in the salt formation the less acidic γ -CO_2H group plays an important role in the stabilization of transition state leading to the formation of α -activated ester via hydrogen bonding.

The results of HPLC as well as via isolation of products by tlc clearly show that the major preference for dipeptide formation involves the α-CO_2H function of Glu and the amino grouping of the Leu leading to BzGlu(γ -OMe)-LeuOMe (32). This again may be a reflection of the higher basicity of the NH_2 function of the Leu (pka 9.74) compared to that of Glu (pka 9.47). The very low preference exhibited in the formation of dipeptide Bzleu-leu-OMe (30) (8%) is quite surprising. Equally surprising is the fact that excepting in the case of BzleuleuOMe (30), the Leu residue does not appear at the amino end of the dipeptide. This factor perhaps supports the illustration provided in CHART C.15 wherein the γ -CO_2H group plays a dominant role. This aspect has been clearly brought out from blank reactions where Glu and Leu were separately reacted with equivalent amounts of the water soluble carbodiimide (22) for 2 days, the reaction mixture N,C-protected and analysed by HPLC.

In the case of Glu, HPLC showed as relative percentages BzGlu (α -OMe)-GludiOMe (34), (54.6%) and BzGlu(γ -OMe)GludiOMe (33), (44.6%)

CHART C-15



and a negligible amount of 0.475% of BzGlu-diOMe arising from recovery of starting material.

The reasons for the formation of almost equal amounts of α and γ peptides in this experiment in contrast to the case where Glu had to compete with Leu for (22) is not clear.

In sharp contrast, with Leucine HPLC showed as relative percentages Bzleu-leu-OMe (30) (36.5%), BzleuOMe (32.4%) arising from non-participation in peptide bond formation - and peak tentatively assigned to tripeptide Bzleu-leu-leu-OMe (29.3%). In terms of actual isolated yields, Leucine gave after reaction with (22) as described above followed by N,C-protection and preparative tlc 21% of BzleuOMe and 20% of Bzleu-leu-OMe (30).

The single experiment involving the reaction of Glu and Leu in presence of the water soluble carbodiimide (22) in H_2O, supported by a number of ancillary experiments have provided numerous facets associated with peptide bond formation. Thus, the selectivity observed could be a result of a number of properties.

Similar studies involving Glycine in place of Leucine was considered important since such study could bring about the consequences arising from steric factors with respect to peptide bond formation. As in the previous example the authentic samples of all the possible products were prepared by condensation of the appropriate partners using DCC-HOBt in methylene chloride rt. The 4 additional dipeptides prepared were, BzGly-Gly-OMe (37) (56%) BzGlyGlu-diOMe (38) (62%), BzGlu(γ-OMe)GlyOMe (35) (34%) and BzGlu(α -OMe)-GlyOMe (36) (52%).

BzGlu(γ -OMe)-Gly-OMe (35) :

Thick syrup

ir : ν_{max}(neat) cm^{-1} : 3340, 3280 (-NH), 1750 (ester), 1650, 1540 (amide).

nmr : δ ($CDCl_3$) : 2.1 - 2.8 (m, 4H, methylenes), 3.6 (s, 3H, γ -$COO\underline{CH_3}$), 3.7 (s, 3H, α -$COO\underline{CH_3}$), 4.0 (d, 2H, NH-$C\underline{H_2}$-COOMe), 4.8 (m, 1H, tert.proton), 7.2 - 7.9 (m, 7H, aromatic + 2 x NH).

ms : m/z : 248 (M^+ -$NHCH_2COOMe$), 220 ($BzNHCHCH_2CH_2CO_2Me$).

BzGlu(α -OMe)Gly-OMe (36) :

mp. 101°C.

ir : ν_{max}(KBr) cm^{-1} : 3320 (-NH), 1740 (ester), 1640, 1540 (amide).

nmr : δ ($CDCl_3$) : 1.9 - 2.5 (m, 4H, methylenes), 3.65 (s, 3H, α - $COO\underline{CH_3}$), 3.7 (d, 2H, $NHC\underline{H_2}$-COOMe), 3.8 (s, 3H, α-$COO\underline{CH_3}$), 4.6 (m, 1H, tert.proton), 7.3 - 7.8 (m, 5H, aromatic).

ms : m/z : 143 $(M+H - BzNHCH_2COOMe)^+$.

BzGly-Gly-OMe (37) :

mp. 111-112°C.

ir : ν_{max}(KBr) cm^{-1} : 3290 (NH), 1730 (ester), 1540, 1640 (amide).

nmr : δ ($CDCl_3$) : 3.7 (s, 3H, $COO\underline{Me}$), 4.2 (d, d, 4H, $C\underline{H_2}C\underline{H_2}$), 7.3 -7.9 (m, 5H, aromatic).

ms : 162 ($BzNHCH_2CO)^+$, 134 ($BzNHCH_2)^+$.

BzGly-Glu(γ -OMe)-OMe (38) :

mp. 107°C.

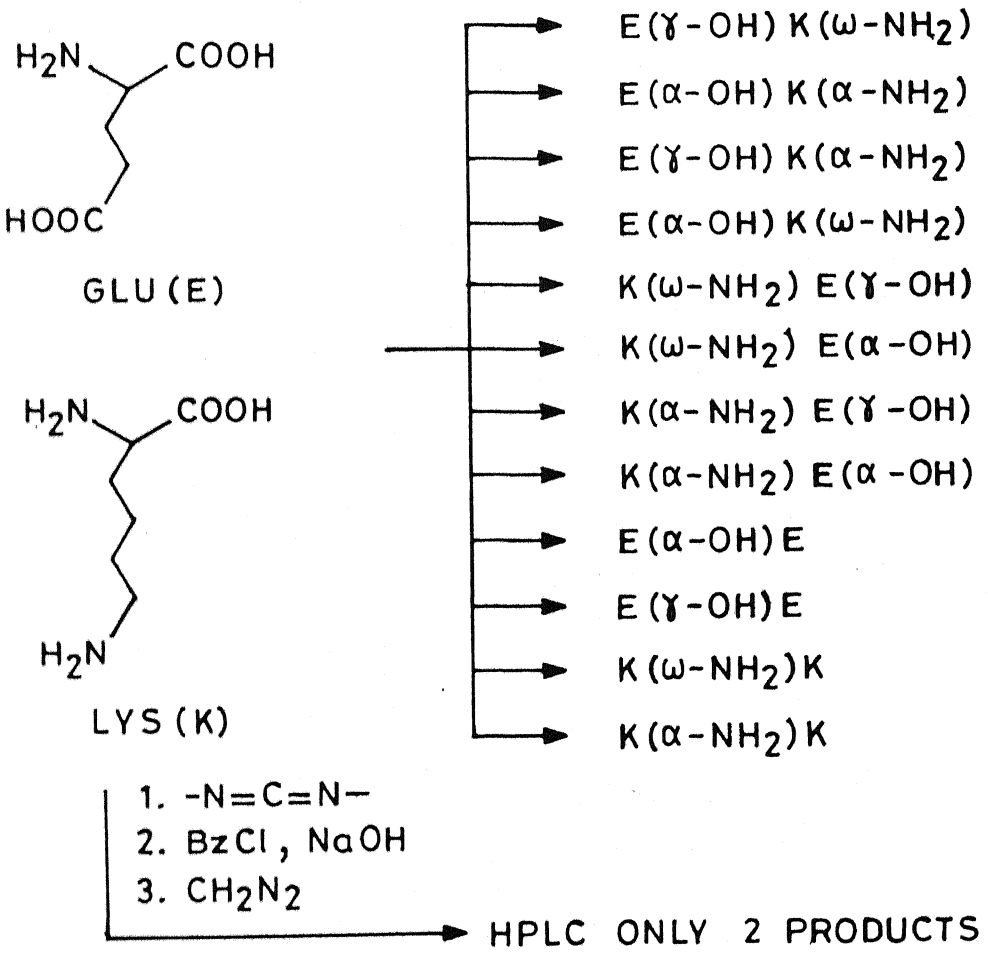
ir : ν_{max}(KBr) cm^{-1} : 3280 (NH), 1740 (ester), 1660, 1630, 1540 (amide).

nmr : δ($CDCl_3$) : 1.9 - 2.6 (m, 4H, -$CHCH_2$-CH_2COOMe), 3.6 (s, 3H, γ -$COOCH_3$), 3.7 (s, 3H, α -$COOCH_3$), 4.2 (d, 2H, NH-CH_2), 4.6 (m, 1H, tert.proton), 7.2 - 7.9 (m,. 7H, aromatic + 2 x NH).

The correlation of reactivity towards water soluble carbodiimide, of amino acid side chains, finds dramatic expression in the reaction of equivalent amounts of Glutamic acid, Glycine and the water soluble carbodiimide (22) in H_2O for two days. HPLC analysis of the reaction mixture after N,C-protection showed that the product consisted entirely of Gly-Gly and higher peptides. This observation is also supported via blank experiment involving Glycine and (22) as well as by the nmr exhibited by the product in the Glu-Gly experiment. Thus, in terms of activated ester formation and in the peptide bond formation, Glycine exerts an overwhelming influence.

The experimental complexities associated with the possible analysis of dipeptides arising from two amino acids in aqueous medium is maximum in the case of the reaction of a dicarboxylic acid like Glutamic acid with a dibasic acid like Lysine. This could, in principle, as illustrated in CHART C.16 result in 12 dipeptides. In the event, the reaction of Glutamic acid and Lysine in clear water solution with 1 equivalent of water soluble carbodiimide followed

CHART C-16


H2N
COOH
HOOC
GLU (E)
H2N
COOH
H2N
LYS (K)
E(γ-OH) K(ω-NH2)
E(α-OH) K(α-NH2)
E(γ-OH) K(α-NH2)
E(α-OH) K(ω-NH2)
K(ω-NH2) E(γ-OH)
K(ω-NH2) E(α-OH)
K(α-NH2) E(γ-OH)
K(α-NH2) E(α-OH)
E(α-OH) E
E(γ-OH) E
K(ω-NH2) K
K(α-NH2) K
1. -N=C=N-
2. BzCl, NaOH
3. CH2N2
HPLC ONLY 2 PRODUCTS

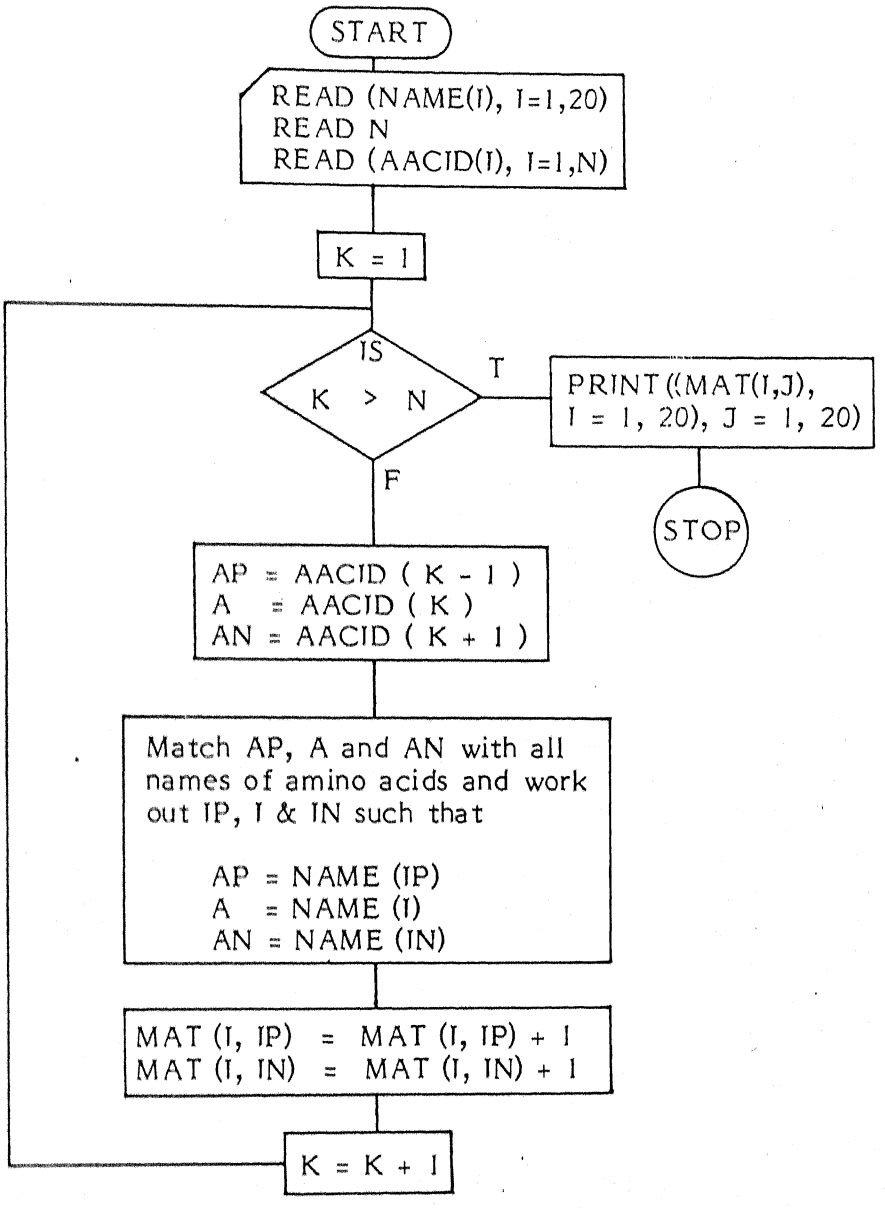
by N,C-protection and HPLC analysis showed only two products. Regardless of the nature of these products which remains to be established, this experiment clearly showed a high order of selectivity in peptide bond formation. Parenthetically, equivalent amounts of Lysine and (22) followed by N,C-protection demonstrated formation of Bz Lys(ω -Bz)lys-(ω -Bz)-OMe based on HPLC and NMR. The above sets of experiments although comprising of minimum complexity supports strongly the notion that if peptide bonds permitted to be formed in a amino acid milieu, a very high order of selectivity can be anticipated.

As stated earlier, it was considered worthwhile to complement the experimental results which really establish selectivity in peptide bond formation with information available in the form of amino acid sequences of enzymes and proteins. At the outset, it was considered that the best methodology for the analysis of the large data base available, would be via development of a computer program that has the capability to identify neighbours present in each of the amino acid residues that comprise the enzyme. Details pertaining to this program are briefly presented in SCHEME C-1.

Such a computer based analysis of a representative data base comprising of Cytochrome of 16 different species about differences pertaining to the choice of the neighbours was carried out.

If it is assumed that a particular coded amino acid occurs n-times in a protein it could have 2n neighbours if the residue is non-terminal and 2n-1 if terminal(Nbr).If these slots are to be allocated without selectivity it can be expected that the available slots would be uniformly occupied by the 20 coded amino acids. Therefore the probability of a particular coded amino acid being present as the neighbour of n would be $\frac{2n}{20}$ = 0.1 n if n is non-terminal and $\frac{2n-1}{20}$ if terminal(R.V.).Any deviation from these values would therefore be a

SCHEME C-1



NAME	:	Array of dimension 20 which contains names of all amino acids (#20).
N	:	Total number of amino acid present in proteins.
AACID	:	Array of dimension N, stores distribution of 'N' amino acids in proteins.
MAT	:	Matrix of dimension 20 x 20. Value of MAT (I,J) represents number of occurrences when NAME (J) found to be NAME (I) of neighbour.

reflection of selectivity.

The computer based analysis of the Cytochrome family is presented in TABLE C.2 - TABLE C.18. The uniform departure from the random values found in each case and in each of the tables reflect preference in the choice of neighbours. That this finding is not fortuitous is shown by a similar analysis in the larger protein, bovine glutamate dehydrogenase, presented in TABLE C.19. The data presented in TABLE C.2 - TABLE C.19 could be analysed from diverse perspectives.

The diagonal element in each of the above tables denote preferences for having as neighbour another unit of the same amino acid. In the TABLE C.2 - C.18, the diagonal element is zero in 75-80% of the cases, which would indicate a lack of preference for itself, barring few exceptions. Even in the case of the larger protein presented in TABLE C.19, 50% of the diagonal element is zero. There is every reason to believe that this would be the case in more extensive analysis planned with a much larger data base. This anticipation is confirmed by a search for Ser-Ser residues in 150 diverse proteins. The Se-Ser residue was selected for detailed analysis because a casual examination of number of sequences showed that whilst Ser residue is relatively abundant in proteins, the Ser-Ser repetition is rare. The analysis of 150 proteins done manually, is presented in TABLE C.20. An examination of the table showed that by and large, barring notable exception the Ser-Ser observed values are invariably either zero or much below the random values. Thus 96 of the 150 cases analysed show a lower preference to the expected value.

An interesting observation is that whilst in most proteins Ser-Ser combination is rare, in few they are abundant. Further, this abundance is typical and can be directly correlated to specific proteins, such as a) Carbonic anhydrase

TABLE C-2

CYTOCHROME (b562) OF E.COLI

AA	PRED	Ndf	R.V.	ALA	CYS	ASP	GLU	PHE	GLY	HIS	ILE	LYS	LEU	MET	ASN	PRO	GLN	ARG	SER	THR	VAL	TRP	TYR	MIN	MAX
ALA	17	33	1.60	8	0	3	3	0	0	0	0	3	3	0	4	0	5	1	0	1	0	0	1	0	8
CYS	0	0	.00	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ASP	10	20	1.00	3	0	4	2	2	0	0	2	2	1	1	3	0	0	0	0	0	0	0	0	0	4
GLU	7	14	.70	3	0	2	0	0	2	0	2	2	2	0	1	0	0	0	0	0	0	0	0	0	3
PHE	2	4	.20	0	0	2	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	2
GLY	3	6	.30	0	0	0	2	1	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	2
HIS	2	4	.20	0	0	0	0	0	1	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	1
ILE	3	6	.30	0	0	2	2	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	2
LYS	16	32	1.60	3	0	2	2	0	1	1	0	2	5	2	0	1	2	0	1	1	6	0	2	0	6
LEU	9	18	.90	3	0	1	2	0	0	0	1	5	0	0	3	0	1	0	0	1	1	0	0	0	5
MET	3	6	.30	0	0	1	0	0	0	0	0	2	0	0	0	1	1	1	0	0	0	0	0	0	2
ASN	7	14	.70	4	0	3	1	0	0	0	0	0	3	0	0	1	0	1	1	0	0	0	0	0	4
PRO	4	8	.40	0	0	0	0	0	0	0	0	1	0	1	1	2	1	0	1	1	0	0	0	0	2
GLN	7	14	.70	5	0	0	0	0	0	1	0	2	1	1	0	1	0	0	1	1	1	0	0	0	5
ARG	4	7	.30	1	0	0	0	1	0	1	0	0	0	1	1	0	0	0	0	1	0	0	1	0	1
SER	2	4	.20	0	0	0	0	0	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0	1
THR	4	8	.40	1	0	0	0	0	0	0	0	1	1	0	0	1	1	1	0	2	0	0	0	0	2
VAL	5	10	.50	0	0	0	0	0	1	0	1	6	1	0	0	0	1	0	0	0	0	0	0	0	6
TRP	0	0	.00	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TYR	2	4	.20	1	0	0	0	0	0	0	0	2	0	0	0	0	0	1	0	0	0	0	0	0	2

TOTAL 107

Unmatched AMINO ACIDS : 3

TABLE C-3

YEAST CYTOCHROME (b2) CORE

AA	FREQ	Nor	R.V.	ALA	CYS	ASP	GLU	PHE	GLY	HIS	ILE	LYS	LEU	MET	ASN	PRO	GLN	ARG	SER	THR	VAL	TRP	TYR	MIN	MAX
ALA	7	14	.70	0	0	0	1	0	1	1	2	1	0	0	1	4	0	0	0	1	1	0	1	0	4
CYS	2	4	.20	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	1
ASP	5	10	.50	0	1	2	0	0	0	0	0	1	1	0	0	1	1	0	0	0	2	0	1	0	2
GLU	5	10	.50	1	0	0	0	1	2	0	0	0	2	0	0	2	0	0	0	1	1	0	0	0	2
PHE	3	6	.30	0	0	0	1	0	0	0	1	1	1	0	1	0	0	1	0	0	0	0	0	0	1
GLY	7	14	.70	1	0	0	2	0	2	0	0	1	1	0	1	3	1	0	1	0	0	0	1	0	3
HIS	3	6	.30	1	0	0	0	0	0	0	0	1	1	0	2	1	0	0	0	0	0	0	0	0	2
ILE	6	12	.60	2	0	0	0	1	0	0	0	2	0	0	1	0	0	0	1	0	3	0	1	0	3
LYS	9	17	.80	1	0	1	0	1	1	1	2	0	1	0	1	1	3	0	0	1	0	0	1	0	3
LEU	6	12	.60	0	0	1	2	1	1	1	0	1	0	0	0	3	0	0	0	1	1	0	0	0	3
MET	1	2	.10	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1
ASN	4	8	.40	1	0	0	0	1	1	2	1	1	0	0	0	1	0	0	0	0	0	0	0	0	2
PRO	13	26	1.30	4	1	1	2	0	3	1	0	1	3	1	1	4	1	0	1	0	0	0	1	0	4
GLN	3	6	.30	0	0	1	0	0	1	0	0	3	0	0	0	1	0	0	0	0	0	0	0	0	3
ARG	1	2	.10	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1
SER	2	4	.20	0	0	0	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	1
THR	3	6	.30	1	0	0	1	0	0	0	0	1	1	0	0	0	0	1	0	0	1	0	0	0	1
VAL	8	16	.80	1	1	2	1	0	0	0	3	0	1	0	0	0	0	0	0	1	2	1	2	0	3
TRP	1	2	.10	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
TYR	1	2	.40	1	0	1	0	0	1	0	1	1	0	0	0	1	0	0	0	0	2	0	0	0	2

TOTAL 93

Unmatched AMINO ACIDS : 2

TABLE C-4

BUCKWHEAT CYTOCHROME (C)

AA	FREQ	Nor	R.V.	ALA	CYS	ASP	GLU	PHE	GLY	HIS	ILE	LYS	LEU	MET	ASN	PRO	GLN	ARG	SER	THR	VAL	TRP	TYR	MIN	MAX
ALA	10	20	1.00	2	1	1	1	0	3	0	1	1	0	0	1	1	1	1	1	2	1	0	1	0	3
CYS	2	4	.20	1	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1
ASP	2	4	.20	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1
GLU	6	12	.60	1	0	1	0	0	2	0	0	2	0	0	0	0	1	1	1	0	1	0	2	0	2
PHE	4	8	.40	0	0	0	0	0	1	0	1	1	1	0	0	1	0	0	1	1	1	0	0	0	1
GLY	12	24	1.20	3	0	0	2	1	0	1	0	1	2	0	2	4	1	1	2	2	0	1	1	0	4
HIS	2	4	.20	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1
ILE	4	8	.40	1	0	0	0	1	0	0	0	2	1	0	1	1	0	0	0	0	0	0	1	0	2
LYS	12	24	1.20	1	1		2	1	1	1	2	0	1	1	4	1	1	0	1	3	0	0	1	0	4
LEU	8	16	.80	0	0	1	0	1	2	0	1	1	2	0	3	0	0	0	0	1	0	0	3	0	3
MET	1	2	.10	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1
ASN	7	14	.70	1	0	0	0	0	2	0	1	4	3	0	0	2	0	0	1	0	0	0	0	0	4
PRO	7	14	.70	1	0	0	0	1	4	0	1	1	0	0	2	2	1	0	0	0	0	0	0	0	4
GLN	5	10	.50	1	1	0	1	0	1	0	0	1	0	0	0	1	0	1	2	0	0	0	0	0	2
ARG	2	4	.20	1	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
SER	6	12	.60	1	0	0	1	1	2	0	0	1	0	0	1	0	2	0	0	0	0	0	3	0	3
THR	8	16	.80	2	0	1	0	1	2	1	0	3	1	0	0	0	0	0	0	2	2	1	0	0	3
VAL	3	6	.30	1	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	2	0	0	0	0	2
TRP	1	2	.10	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
TYR	5	12	.60	1	0	0	2	0	1	0	1	1	3	0	0	0	0	0	3	0	0	0	0	0	3

TOTAL 108

Unmatched AMINO ACIDS : 4

TABLE C-5

WHEAT GERM CYTOCHROME (c)

AA	FREQ	Nor	R.V.	ALA	CYS	ASP	GLU	PHE	GLY	HIS	ILE	LYS	LEU	MET	ASN	PRO	GLN	ARG	SER	THR	VAL	TRP	TYR	MIN	MAX
ALA	14	28	1.40	2	1	3	1	0	6	0	1	3	0	0	1	1	1	1	2	2	1	0	1	0	6
CYS	2	4	.20	1	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1
ASP	5	10	.50	3	0	0	0	0	0	0	0	0	1	0	0	1	1	1	0	0	1	0	2	0	3
GLU	4	8	.40	1	0	0	2	0	0	0	0	0	0	0	1	0	0	0	1	0	1	2	0	0	2
PHE	4	8	.40	0	0	0	0	0	1	0	1	1	1	0	0	1	0	0	2	0	1	0	0	0	2
GLY	11	22	1.10	6	0	0	0	1	0	2	0	0	2	0	1	4	1	1	1	2	0	0	1	0	6
HIS	3	6	.30	0	1	0	0	0	2	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	2
ILE	3	6	.30	1	0	0	0	1	0	0	0	1	1	0	0	1	0	0	0	0	0	0	1	0	1
LYS	13	26	1.30	3	1	0	0	1	0	1	1	6	2	1	3	2	1	0	0	3	0	0	1	0	6
LEU	8	16	.80	0	0	1	0	1	2	1	1	2	2	0	2	0	0	0	0	1	0	0	3	0	3
MET	1	2	.10	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1
ASN	6	12	.60	1	0	0	1	0	1	0	0	3	2	0	0	3	0	0	0	1	0	0	0	0	3
PRO	8	16	.80	1	0	1	0	1	4	0	1	2	0	0	3	2	1	0	0	0	0	0	0	0	4
GLN	4	8	.40	1	1	1	0	0	1	0	0	1	0	0	0	1	0	1	1	0	0	0	0	0	1
ARG	2	4	.20	1	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
SER	7	13	.60	2	0	0	1	2	1	0	0	0	0	0	0	0	1	0	2	1	0	0	3	0	3
THR	7	14	.70	2	0	0	0	0	2	1	0	3	1	0	1	0	0	0	1	2	1	0	0	0	3
VAL	3	6	.30	1	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1
TRP	1	2	.10	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
TYR	6	12	.60	1	0	2	0	0	1	0	1	1	3	0	0	0	0	0	3	0	0	0	0	0	3

TOTAL 112

Unmatched AMINO ACIDS : 1

TABLE C-6

CYTOCHROME c-BLOWFLY

AA	FREQ	Nbr	R.V.	ALA	CYS	ASP	GLU	PHE	GLY	HIS	ILE	LYS	LEU	MET	ASN	PRO	GLN	ARG	SER	THR	VAL	TRP	TYR	MIN	MAX
ALA	10	20	1.00	0	1	0	1	2	3	0	1	2	0	0	2	2	2	0	1	1	0	0	2	0	3
CYS	2	4	.20	1	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1
ASP	4	8	.40	0	0	2	0	0	2	0	0	0	1	0	0	0	1	0	0	1	1	0	0	0	2
GLU	5	10	.50	1	0	0	0	1	0	0	0	1	1	0	2	0	0	1	0	0	2	0	1	0	2
PHE	5	10	.50	2	0	0	1	0	2	0	2	0	2	0	0	0	0	0	0	0	1	0	0	0	2
GLY	14	27	1.30	3	0	2	0	2	2	1	1	4	2	0	0	3	1	2	0	2	2	0	0	0	4
HIS	3	6	.30	0	1	0	0	0	1	0	0	2	1	0	0	0	0	0	0	1	0	0	0	0	2
ILE	5	10	.50	1	0	0	0	2	1	0	0	1	1	1	0	1	0	0	0	1	0	0	1	0	2
LYS	15	29	1.40	2	0	0	1	0	4	2	1	6	2	1	1	2	0	1	1	3	1	0	1	0	6
LEU	7	14	.70	0	0	1	1	2	2	1	1	2	0	0	1	0	0	0	0	1	0	0	2	0	2
MET	1	2	.10	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1
ASN	5	10	.50	2	0		2	0	0	0	0	1	1	0	0	3	0	0	0	1	0	0	0	0	3
PRO	6	12	.60	2	0	0	0	0	3	0	1	2	0	0	3	0	0	0	0	0	1	0	0	0	3
GLN	4	8	.40	2	1	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	2
ARG	3	6	.30	0	1	0	1	0	2	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	2
SER	1	2	.10	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1
THR	7	14	.70	1	0	1	0	0	2	1	1	3	1	0	1	0	0	0	0	0	1	1	1	0	3
VAL	5	10	.50	0	0	1	2	1	2	0	0	1	0	0	0	1	1	0	0	1	0	0	0	0	2
TRP	1	2	.10	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1
TYR	4	8	.40	2	0	0	1	0	0	0	1	1	2	0	0	0	0	0	0	1	0	0	0	0	2

TOTAL 107

Unmatched AMINO ACIDS : 0

TABLE C-7

CYTOCHROME c-LOCUST

AA	FREQ	Nbr	R.V.	ALA	CYS	ASP	GLU	PHE	GLY	HIS	ILE	LYS	LEU	MET	ASN	PRO	GLN	ARG	SER	THR	VAL	TRP	TYR	MIN	MAX
ALA	11	22	1.10	2	1	*	1	2	4	0	1	2	0	0	1	1	2	0	1	1	0	0	2	0	4
CYS	2	4	.20	1	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1
ASP	4	8	.10	1	0	0	1	0	2	0	0	0	1	0	0	0	0	0	0	2	1	0	0	0	2
GLU	6	12	.60	1	0	1	0	1	0	0	0	1	1	0	3	0	0	1	0	0	2	0	1	0	3
PHE	4	8	.40	2	0	0	1	0	1	0	1	0	2	0	0	0	0	0	0	0	1	0	0	0	2
GLY	14	27	1.30	4	0	2	0	1	2	1	2	4	2	0	0	2	1	2	0	2	2	0	0	0	4
HIS	3	6	.30	0	1	0	0	0	1	0	0	2	1	0	0	0	0	0	0	1	0	0	0	0	2
ILE	5	10	.50	1	0	0	0	1	2	0	0	0	2	1	0	1	0	0	0	1	0	0	1	0	2
LYS	15	29	1.40	2	0	0	1	0	4	2	0	6	3	1	1	2	0	1	1	3	1	0	1	0	6
LEU	8	16	.80	0	0	1	1	2	2	1	2	3	0	0	1	0	0	0	0	1	0	0	2	0	3
MET	1	2	.10	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1
ASN	5	10	.50	1	0	0	3	0	0	0	0	1	1	0	0	3	0	0	0	0	0	1	0	0	3
PRO	5	10	.50	1	0	0	0	0	2	0	1	2	0	0	3	0	0	0	0	0	1	0	0	0	3
GLN	3	6	.30	2	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	2
ARG	3	6	.30	0	1	0	1	0	2	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	2
SER	1	2	.10	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1
THR	7	14	.70	1	0	2	0	0	2	1	1	3	1	0	0	0	0	0	0	0	1	1	1	0	3
VAL	5	10	.50	0	0	1	2	1	2	0	0	1	0	0	0	1	1	0	0	1	0	0	0	0	2
TRP	1	2	.10	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1
TYR	4	8	.40	2	0	0	1	0	0	0	1	1	2	0	0	0	0	0	0	1	0	0	0	0	2

TOTAL 107

Unmatched AMINO ACIDS : 0

TABLE C-8

CYTOCHROME c-INSECT CERATITIS CAPITATA

AA	FREQ	Nor	R.V.	ALA	CYS	ASP	GLU	PHE	GLY	HIS	ILE	LYS	LEU	MET	ASN	PRO	GLN	ARG	SER	THR	VAL	TRP	TYR	MIN	MAX
ALA	11	22	1.10	2	1	1	1	2	4	0	1	2	0	0	1	1	2	0	1	1	0	0	2	0	4
CYS	2	4	.20	1	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1
ASP	4	8	.40	1	0	0	1	0	2	0	0	0	1	0	0	0	0	0	0	2	1	0	0	0	2
GLU	6	12	.60	1	0	1	0	1	0	0	0	1	1	0	3	0	0	1	0	0	2	0	1	0	3
PHE	4	8	.40	2	0	0	1	0	1	0	1	0	2	0	0	0	0	0	0	0	1	0	0	0	2
GLY	14	27	1.30	4	0	2	0	1	2	1	2	4	2	0	0	2	1	2	0	2	2	0	0	0	4
HIS	3	6	.30	0	1	0	0	0	1	0	0	2	1	0	0	0	0	0	0	1	0	0	0	0	2
ILE	5	10	.50	1	0	0	0	1	2	0	0	0	2	1	0	1	0	0	0	1	0	0	1	0	2
LYS	15	29	1.40	2	0	0	1	0	4	2	0	6	3	1	1	2	0	1	1	3	1	0	1	0	6
LEU	8	16	.80	0	0	1	1	2	2	1	2	3	0	0	1	0	0	0	0	1	0	0	2	0	3
MET	1	2	.10	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1
ASN	5	10	.50	1	0	0	3	0	0	0	0	1	1	0	0	3	0	0	0	0	0	1	0	0	3
PRO	5	10	.50	1	0	0	0	0	2	0	1	2	0	0	3	0	0	0	0	0	1	0	0	0	3
GLN	3	6	.30	2	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	2
ARG	3	6	.30	0	1	0	1	0	2	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	2
SER	1	2	.10	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1
THR	7	14	.70	1	0	2	0	0	2	1	1	3	1	0	0	0	0	0	0	0	1	1	1	0	3
VAL	5	10	.50	0	0	1	2	1	2	0	0	1	0	0	0	1	1	0	0	1	0	0	0	0	2
TRP	1	2	.10	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1
TYR	4	8	.40	2	0	0	1	0	0	0	1	1	2	0	0	0	0	0	0	1	0	0	0	0	2

TOTAL 107

Unmatched AMINO ACIDS : 0

TABLE C-9

TOBACCO MOTH CYTOCHROME (c)

AA	FREQ	Nor	R.V.	ALA	CYS	ASP	GLU	PHE	GLY	HIS	ILE	LYS	LEU	MET	ASN	PRO	GLN	ARG	SER	THR	VAL	TRP	TYR	MIN	MAX
ALA	12	24	1.20	0	1	2	1	1	3	0	1	3	0	0	4	2	3	1	0	1	0	0	1	0	4
CYS	2	4	.20	1	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1
ASP	4	8	.40	2	0	2	0	0	0	0	0	0	1	0	1	0	1	0	0	1	0	0	0	0	2
GLU	4	8	.40	1	0	0	0	1	0	0	0	0	1	0	2	0	0	1	0	0	1	0	1	0	2
PHE	6	12	.60	1	0	0	1	2	3	0	1	0	1	0	0	0	0	0	1	0	2	0	0	0	3
GLY	13	25	1.20	3	0	0	0	3	2	1	1	3	1	0	2	3	1	1	0	2	2	0	0	0	3
HIS	3	6	.30	0	1	0	0	0	1	0	0	2	1	0	0	0	0	0	0	1	0	0	0	0	2
ILE	4	8	.40	1	0	0	0	1	1	0	0	1	1	0	0	1	0	0	0	1	0	0	1	0	1
LYS	14	27	1.30	3	0	0	0	0	3	2	1	6	2	1	1	1	1	1	0	3	1	0	1	0	6
LEU	6	12	.60	0	0	1	1	1	1	1	1	2	0	0	1	0	0	0	0	1	0	0	2	0	2
MET	1	2	.10	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1
ASN	7	14	.70	4	0	1	2	0	2	0	0	1	1	0	0	2	0	0	1	0	0	0	0	0	4
PRO	5	10	.50	2	0	0	0	0	3	0	1	1	0	0	2	0	0	0	0	0	1	0	0	0	3
GLN	5	10	.50	3	1	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0	1	1	0	0	3
ARG	3	6	.30	1	1	0	1	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1
SER	2	4	.20	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	2	0	2
THR	6	12	.60	1	0	1	0	0	2	1	1	3	1	0	0	0	0	0	0	0	1	1	0	0	3
VAL	5	10	.50	0	0	0	1	2	2	0	0	1	0	1	0	1	1	0	0	1	0	0	0	0	2
TRP	1	2	.10	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1
TYR	4	8	.40	1	0	0	1	0	0	0	1	1	2	0	0	0	0	0	2	0	0	0	0	0	2

TOTAL 107

Unmatched AMINO ACIDS : 0

TABLE C-10

SILKWORM CYTOCHROME (c)

AA	FREQ	Ndr	R.V.	ALA	CYS	ASP	GLU	PHE	GLY	HIS	ILE	LYS	LEU	MET	ASN	PRO	GLN	ARG	SER	THR	VAL	TRP	TYR	MIN	MAX
ALA	11	22	1.10	0	1	1	2	1	3	0	1	3	0	0	4	2	2	1	0	0	0	0	1	0	4
CYS	2	4	.20	1	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1
ASP	3	6	.30	1	0	2	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	2
GLU	6	12	.60	2	0	0	0	1	0	0	0	1	1	0	3	0	0	1	1	0	1	0	1	0	3
PHE	5	10	.50	1	0	0	1	0	2	0	1	0	1	0	0	0	0	0	1	0	2	0	1	0	2
GLY	14	27	1.30	3	0	1	0	2	2	1	1	3	1	0	2	3	1	1	0	2	2	1	1	0	3
HIS	3	6	.30	0	1	0	0	0	1	0	0	2	1	0	0	0	0	0	0	1	0	0	0	0	2
ILE	4	8	.40	1	0	0	0	1	1	0	0	1	1	0	0	1	0	0	0	1	0	0	1	0	1
LYS	14	27	1.30	3	0	0	1	0	3	2	1	6	2	1	1	1	0	1	0	3	1	0	1	0	6
LEU	6	12	.60	0	0	1	1	1	1	1	1	2	0	0	1	0	0	0	0	1	0	0	2	0	2
MET	1	2	.10	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1
ASN	7	14	.70	4	0	0	3	0	2	0	0	1	1	0	0	2	0	0	1	0	0	0	0	0	4
PRO	5	10	.50	2	0	0	0	0	3	0	1	1	0	0	2	0	0	0	0	0	1	0	0	0	3
GLN	3	6	.30	2	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	2
ARG	3	6	.30	1	1	0	1	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1
SER	3	6	.30	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	2	0	2
THR	6	12	.60	0	0	1	0	0	2	1	1	3	1	0	0	0	0	0	1	0	1	1	0	0	3
VAL	5	10	.50	0	0	0	1	2	2	0	0	1	0	1	0	1	1	0	0	1	0	0	0	0	2
TRP	1	2	.10	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
TYR	5	10	.50	1	0	0	1	1	1	0	1	1	2	0	0	0	0	0	2	0	0	0	0	0	2

TOTAL 107

Unmatched AMINO ACIDS : 0

TABLE C-11

CYTOCHROME c-GUANACO

AA	FREQ	Nor	R.V.	ALA	CYS	ASP	GLU	PHE	GLY	HIS	ILE	LYS	LEU	MET	ASN	PRO	GLN	ARG	SER	THR	VAL	TRP	TYR	MIN	MAX
ALA	6	12	.60	0	1	1	0	1	1	0	1	1	0	0	1	1	2	0	0	1	0	0	1	0	2
CYS	2	4	.20	1	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1
ASP	3	6	.30	1	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	1
GLU	9	17	.80	0	0	1	2	0	2	0	0	2	1	1	2	0	0	2	0	1	2	0	1	0	2
PHE	4	8	.40	1	0	0	0	0	2	0	2	0	1	0	0	0	0	0	1	0	1	0	0	0	2
GLY	14	28	1.40	1	0	1	2	2	2	1	2	6	1	0	0	3	1	1	0	3	0	1	0	0	6
HIS	3	6	.30	0	1	0	0	0	1	0	0	2	1	0	0	0	0	0	0	1	0	0	0	0	2
ILE	6	12	.60	1	0	0	0	2	2	0	0	2	1	1	0	1	0	0	0	1	0	0	1	0	2
LYS	18	36	1.80	1	1	0	2	0	6	2	2	10	1	1	3	1	1	1	0	3	0	0	1	0	10
LEU	6	12	.60	0	0	1	1	1	1	1	1	1	0	1	1	0	0	0	0	1	0	0	2	0	2
MET	2	4	.20	0	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1
ASN	5	10	.50	1	0	0	2	0	0	0	0	3	1	0	0	2	0	0	0	1	0	0	0	0	3
PRO	4	8	.40	1	0	0	0	0	3	0	1	1	0	0	2	0	0	0	0	0	0	0	0	0	3
GLN	3	6	.30	2	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	2
ARG	2	4	.20	0	0	0	2	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	2
SER	1	2	.10	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
THR	8	16	.80	1	0	1	1	0	3	1	1	3	1	0	1	0	0	0	0	0	1	1	1	0	3
VAL	3	6	.30	0	0	1	2	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	2
TRP	1	2	.10	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
TYR	4	8	.40	1	0	0	1	0	0	0	1	1	2	0	0	0	0	0	1	1	0	0	0	0	2

TOTAL 104

Unmatched AMINO ACIDS : 1

TABLE C-12

RABBIT HEART CYTOCHROME (c)

AA	FREQ	Nor	R.V.	ALA	CYS	ASP	GLU	PHE	GLY	HIS	ILE	LYS	LEU	MET	ASN	PRO	GLN	ARG	SER	THR	VAL	TRP	TYR	MIN	MAX
ALA	7	14	.70	0	1	2	0	1	1	0	1	1	0	0	1	0	2	1	0	1	1	0	1	0	2
CYS	2	4	.20	1	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1
ASP	5	10	.50	2	0	0	2	0	1	0	0	1	1	0	0	0	0	0	0	2	1	0	0	0	2
GLU	7	13	.60	0	0	2	0	0	1	0	0	2	1	1	2	0	0	1	0	0	2	0	1	0	2
PHE	4	8	.40	1	0	0	0	0	2	0	2	0	1	0	0	0	0	0	1	0	1	0	0	0	2
GLY	13	26	1.30	1	0	1	1	2	2	1	2	5	1	0	0	2	1	1	0	3	1	1	0	0	5
HIS	3	6	.30	0	1	0	0	0	1	0	0	2	1	0	0	0	0	0	0	1	0	0	0	0	2
ILE	6	12	.60	1	0	0	0	2	2	0	0	2	1	1	0	1	0	0	0	1	0	0	1	0	2
LYS	18	36	1.80	1	1	1	2	0	5	2	2	10	1	1	3	1	1	1	0	3	0	0	1	0	10
LEU	6	12	.60	0	0	1	1	1	1	1	1	1	0	1	1	0	0	0	0	1	0	0	2	0	2
MET	2	4	.20	0	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1
ASN	5	10	.50	1	0	0	2	0	0	0	0	3	1	0	0	2	0	0	0	1	0	0	0	0	3
PRO	3	6	.30	0	0	0	0	0	2	0	1	1	0	0	2	0	0	0	0	0	0	0	0	0	2
GLN	3	6	.30	2	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	2
ARG	2	4	.20	1	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1
SER	1	2	.10	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
THR	8	16	.80	1	0	2	0	0	3	1	1	3	1	0	1	0	0	0	0	0	1	1	1	0	3
VAL	4	8	.40	1	0	1	2	1	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	2
TRP	1	2	.10	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
TYR	4	8	.40	1	0	0	1	0	0	0	1	1	2	0	0	0	0	0	1	1	0	0	0	0	2

TOTAL 104

Unmatched AMINO ACIDS : 1

TABLE C-13

CAMEL HEART CYTOCHROME (c)

AA	FREQ	Nor	R.V.	ALA	CYS	ASP	GLU	PHE	GLY	HIS	ILE	LYS	LEU	MET	ASN	PRO	GLN	ARG	SER	THR	VAL	TRP	TYR	MIN	MAX
ALA	7	14	.70	0	1	2	0	1	1	0	1	1	0	0	1	0	2	1	0	1	1	0	1	0	2
CYS	2	4	.20	1	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1
ASP	3	6	.30	2	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	2
GLU	8	15	.70	0	0	0	2	0	2	0	0	2	1	1	2	0	0	1	0	1	2	0	1	0	2
PHE	4	8	.40	1	0	0	0	0	2	0	2	0	1	0	0	0	0	0	1	0	1	0	0	0	2
GLY	14	28	1.40	1	0	1	2	2	2	1	2	6	1	0	0	2	1	1	0	3	1	1	0	0	6
HIS	3	6	.30	0	1	0	0	0	1	0	0	2	1	0	0	0	0	0	0	1	0	0	0	0	2
ILE	6	12	.60	1	0	0	0	2	2	0	0	2	1	1	0	1	0	0	0	1	0	0	1	0	2
LYS	18	36	1.80	1	1	0	2	0	6	2	2	10	1	1	3	1	1	1	0	3	0	0	1	0	10
LEU	6	12	.60	0	0	1	1	1	1	1	1	1	0	1	1	0	0	0	0	1	0	0	2	0	2
MET	2	4	.20	0	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1
ASN	5	10	.50	1	0	0	2	0	0	0	0	3	1	0	0	2	0	0	0	1	0	0	0	0	3
PRO	3	6	.30	0	0	0	0	0	2	0	1	1	0	0	2	0	0	0	0	0	0	0	0	0	2
GLN	3	6	.30	2	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	2
ARG	2	4	.20	1	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1
SER	1	2	.10	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
THR	8	16	.80	1	0	1	1	0	3	1	1	3	1	0	1	0	0	0	0	0	1	1	1	0	3
VAL	4	8	.40	1	0	1	2	1	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	2
TRP	1	2	.10	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
TYR	4	8	.40	1	0	0	1	0	0	0	1	1	2	0	0	0	0	0	1	1	0	0	0	0	2

TOTAL 104

Unmatched AMINO ACIDS : 1

TABLE C-14

HORSE HEART CYTOCHROME (c)

AA	FREQ	NOR	R.V.	ALA	CYS	ASP	GLU	PHE	GLY	HIS	ILE	LYS	LEU	MET	ASN	PRO	GLN	ARG	SER	THR	VAL	TRP	TYR	MIN	MAX
ALA	6	12	.60	0	1	1	0	1	1	0	1	1	0	0	1	1	2	0	0	1	0	0	1	0	2
CYS	2	4	.20	1	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1
ASP	3	6	.30	1	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	1
GLU	9	17	.80	0	0	1	2	0	0	0	0	3	1	1	2	0	0	2	0	2	2	0	1	0	3
PHE	4	8	.40	1	0	0	0	0	2	0	2	0	1	0	0	0	0	0	0	1	1	0	0	0	2
GLY	12	24	1.20	1	0	1	0	2	2	1	2	5	1	0	0	3	1	1	0	3	0	0	0	0	5
HIS	3	6	.30	0	1	0	0	0	1	0	0	2	1	0	0	0	0	0	0	1	0	0	0	0	2
ILE	6	12	.60	1	0	0	0	2	2	0	0	2	1	1	0	1	0	0	0	1	0	0	1	0	2
LYS	19	38	1.90	1	1	0	3	0	5	2	2	10	1	1	3	1	1	1	0	4	0	1	1	0	10
LEU	6	12	.60	0	0	1	1	1	1	1	1	1	0	1	1	0	0	0	0	1	0	0	2	0	2
MET	2	4	.20	0	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1
ASN	5	10	.50	1	0	0	2	0	0	0	0	3	1	0	0	2	0	0	0	1	0	0	0	0	3
PRO	4	8	.40	1	0	0	0	0	3	0	1	1	0	0	2	0	0	0	0	0	0	0	0	0	3
GLN	3	6	.30	2	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	2
ARG	2	4	.20	0	0	0	2	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	2
SER	0	0	.00	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
THR	10	20	1.00	1	0	1	2	1	3	1	1	4	1	0	1	0	0	0	0	0	1	1	2	0	4
VAL	3	6	.30	0	0	1	2	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	2
TRP	1	2	.10	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1
TYR	4	8	.40	1	0	0	1	0	0	0	1	1	2	0	0	0	0	0	0	2	0	0	0	0	2

TOTAL 104

Unmatched AMINO ACIDS : 1

TABLE C-15

BOVINE HEART CYTOCHROME (c)

AA	FREQ	NBT	R.V.	ALA	CYS	ASP	GLU	PHE	GLY	HIS	ILE	LYS	LEU	MET	ASN	PRO	GLN	ARG	SER	THR	VAL	TRP	TYR	MIN	MAX
ALA	6	12	.60	0	1	1	0	1	1	0	1	1	0	0	1	1	2	0	0	1	0	0	1	0	2
CYS	2	4	.20	1	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1
ASP	3	6	.30	1	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	1
GLU	9	17	.80	0	0	1	2	0	2	0	0	2	1	1	2	0	0	2	0	1	2	0	1	0	2
PHE	4	8	.40	1	0	0	0	0	2	0	2	0	1	0	0	0	0	0	1	0	1	0	0	0	2
GLY	14	28	1.40	1	0	1	2	2	2	1	2	6	1	0	0	3	1	1	0	3	0	1	0	0	6
HIS	3	6	.30	0	1	0	0	0	1	0	0	2	1	0	0	0	0	0	0	1	0	0	0	0	2
ILE	6	12	.60	1	0	0	0	2	2	0	0	2	1	1	0	1	0	0	0	1	0	0	1	0	2
LYS	18	36	1.80	1	1	0	2	0	6	2	2	10	1	1	3	1	1	1	0	3	0	0	1	0	10
LEU	6	12	.60	0	0	1	1	1	1	1	1	1	0	1	1	0	0	0	0	1	0	0	2	0	2
MET	2	4	.20	0	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1
ASN	5	10	.50	1	0	0	2	0	0	0	0	3	1	0	0	2	0	0	0	1	0	0	0	0	3
PRO	4	8	.40	1	0	0	0	0	3	0	1	1	0	0	2	0	0	0	0	0	0	0	0	0	3
GLN	3	6	.30	2	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	2
ARG	2	4	.20	0	0	0	2	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	2
SER	1	2	.10	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
THR	8	16	.80	1	0	1	1	0	3	1	1	3	1	0	1	0	0	0	0	0	1	1	1	0	3
VAL	3	6	.30	0	0	1	2	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	2
TRP	1	2	.10	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
TYR	4	8	.40	1	0	0	1	0	0	0	1	1	2	0	0	0	0	0	1	1	0	0	0	0	2

TOTAL 104

Unmatched AMINO ACIDS : 1

TABLE C-16

CHICKEN LIVER MICROSOMAL CYTOCHROME (B5)

AA	FREQ	Nor	R.V.	ALA	CYS	ASP	GLU	PHE	GLY	HIS	ILE	LYS	LEU	MET	ASN	PRO	GLN	ARG	SER	THR	VAL	TRP	TYR	MIN	MAX
ALA	4	8	.40	0	0	2	0	0	1	0	0	0	1	0	0	0	1	2	0	1	0	0	0	0	2
CYS	0	0	.00	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ASP	7	14	.70	2	0	2	2	0	1	0	1	1	1	0	0	1	0	0	0	1	1	0	1	0	2
GLU	9	18	.90	0	0	2	2	1	2	1	0	0	1	0	1	0	2	1	1	2	2	0	0	0	2
PHE	3	6	.30	0	0	0	1	0	0	0	1	1	1	0	1	0	0	0	0	1	0	0	0	0	1
GLY	7	13	.60	1	0	1	2	0	4	1	1	0	0	0	0	1	0	1	0	0	1	0	0	0	4
HIS	6	12	.60	0	0	0	1	0	1	2	0	1	1	0	1	2	0	1	1	0	1	0	0	0	2
ILE	6	12	.60	0	0	1	0	1	1	0	4	0	0	0	0	0	0	1	0	1	1	1	1	0	4
LYS	3	6	.30	0	0	1	0	1	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1
LEU	5	10	.50	1	0	1	1	1	0	1	0	0	0	0	0	0	1	2	1	0	1	0	0	0	2
MET	0	0	.00	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ASN	3	6	.30	0	0	0	1	1	0	1	0	0	0	0	2	0	0	0	1	0	0	0	0	0	2
PRO	3	6	.30	0	0	1	0	0	1	2	0	1	0	0	0	0	0	1	0	0	0	0	0	0	2
GLN	3	6	.30	1	0	0	2	0	0	0	0	0	1	0	0	0	0	0	2	0	0	0	0	0	2
ARG	6	11	.50	2	0	0	1	0	1	1	1	0	2	0	0	1	0	0	0	0	0	0	2	0	2
SER	4	8	.40	0	0	0	1	0	0	1	0	0	1	0	1	0	2	0	0	2	0	0	0	0	2
THR	5	10	.50	1	0	1	2	1	0	0	1	1	0	0	0	0	0	0	0	2	0	0	1	0	2
VAL	4	8	.40	0	0	1	2	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	2
TRP	1	2	.10	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1
TYR	3	6	.30	0	0	1	0	0	0	0	1	0	0	0	0	0	0	2	0	0	0	0	2	0	2

TOTAL 82

Unmatched AMINO ACIDS : 1

TABLE C-17

MONKEY LIVER MICROSOMAL CYTOCHROME (RS)

AA	FREQ	Nbr	R.V.	ALA	CYS	ASP	GLU	PHE	GLY	HIS	ILE	LYS	LEU	MET	ASN	PRO	GLN	ARG	SER	THR	VAL	TRP	TYR	MIN	MAX
ALA	4	8	.40	0	0	2	1	0	1	0	0	0	0	0	0	0	1	1	0	1	1	0	0	0	2
CYS	0	0	.00	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ASP	7	14	.70	2	0	2	2	0	1	0	0	1	1	0	0	1	0	0	1	1	1	0	1	0	2
GLU	11	22	1.10	1	0	2	4	1	2	1	1	0	3	0	1	0	2	2	0	1	1	0	0	0	4
PHE	2	4	.20	0	0	0	1	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	1
GLY	6	12	.60	1	0	1	2	0	4	1	1	0	0	0	0	1	0	0	0	0	1	0	0	0	4
HIS	7	14	.70	0	0	0	1	0	1	2	0	2	2	0	2	2	0	0	2	0	0	0	0	0	2
ILE	4	8	.40	0	0	0	1	0	1	0	2	0	2	0	0	0	0	0	0	0	0	0	1	0	2
LYS	7	14	.70	0	0	1	0	1	0	2	0	0	0	0	0	1	0	0	3	2	2	0	1	0	3
LEU	8	16	.80	0	0	1	3	1	0	2	2	0	0	0	0	0	1	1	1	2	1	1	0	0	3
MET	0	0	.00	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ASN	2	4	.20	0	0	0	1	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
PRO	3	6	.30	0	0	1	0	0	1	2	0	1	0	0	0	0	0	1	0	0	0	0	0	0	2
GLN	2	4	.20	1	0	0	2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	2
ARG	3	5	.20	1	0	0	2	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	2
SER	5	9	.40	0	0	1	0	0	0	2	0	3	1	0	0	0	0	0	0	2	0	0	0	0	3
THR	6	12	.60	1	0	1	1	0	0	0	0	2	2	0	0	0	0	0	2	0	0	1	2	0	2
VAL	4	8	.40	1	0	1	1	0	1	0	0	2	1	0	0	0	0	0	0	0	0	0	1	0	2
TRP	1	2	.10	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1
TYR	4	8	.40	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	2	1	0	2	0	2

TOTAL 86

Unmatched AMINO ACIDS : 1

TABLE C-18

HUMAN LIVER MICROSOMAL CYTOCHROME (85)

AA	FREQ	Nor	R.V.	ALA	CYS	ASP	GLU	PHE	GLY	HIS	ILE	LYS	LEU	MET	ASN	PRO	GLN	ARG	SER	THR	VAL	TRP	TYR	MIN	MAX
ALA	4	8	.40	0	0	-	1	0	1	0	0	0	0	0	0	0	1	1	0	1	1	0	0	0	2
CYS	0	0	.00	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ASP	7	14	.70	2	0	2	2	0	1	0	0	1	1	0	0	1	0	0	1	1	1	0	1	0	2
GLU	11	22	1.10	1	0	2	4	1	2	1	1	0	2	1	1	0	2	2	0	1	1	0	0	0	4
PHE	3	6	.30	0	0	0	1	0	0	0	1	1	1	0	1	0	0	0	0	1	0	0	0	0	1
GLY	6	12	.60	1	0	1	2	0	4	1	1	0	0	0	0	1	0	0	0	0	1	0	0	0	4
HIS	7	14	.70	0	0	0	1	0	1	2	0	2	2	0	2	2	0	0	2	0	0	0	0	0	2
ILE	4	8	.40	0	0	0	1	1	1	0	2	0	2	0	0	0	0	0	0	0	0	0	0	0	2
LYS	7	14	.70	0	0	1	0	1	0	2	0	0	0	0	0	1	0	0	3	2	2	0	1	0	3
LEU	7	14	.70	0	0	1	2	1	0	2	2	0	0	0	0	0	1	1	0	2	1	1	0	0	2
MET	1	2	.10	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
ASN	2	4	.20	0	0	0	1	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
PRO	3	6	.30	0	0	1	0	0	1	2	0	1	0	0	0	0	0	1	0	0	0	0	0	0	2
GLN	2	4	.20	1	0	0	2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	2
ARG	3	5	.20	1	0	0	2	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	2
SER	5	9	.40	0	0	1	0	0	0	2	0	3	0	1	0	0	0	0	0	2	0	0	0	0	3
THR	6	12	.60	1	0	1	1	1	0	0	0	2	2	0	0	0	0	0	2	0	0	1	1	0	2
VAL	4	8	.40	1	0	1	1	0	1	0	0	2	1	0	0	0	0	0	0	0	0	0	1	0	2
TRP	1	2	.10	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1
TYR	3	6	.30	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	2	0	2

TOTAL 86

Unmatched AMINO ACIDS : 1

TABLE C-19

BOVINE GLUTAMATE DEHYDROGENASE

AA	FREQ	Nor	R.V.	ALA	CYS	ASP	GLU	PHE	GLY	HIS	ILE	LYS	LEU	MET	ASN	PRO	GLN	ARG	SER	THR	VAL	TRP	TYR	MIN	MAX
ALA	37	73	3.60	4	2	3	3	2	8	2	3	9	4	1	6	4	2	2	8	3	4	0	3	0	9
CYS	6	12	.60	2	0	2	0	0	0	0	0	3	0	0	1	2	0	0	0	0	2	0	0	0	3
ASP	29	58	2.90	3	2	1	7	2	4	0	4	4	5	0	0	4	1	5	2	3	7	0	1	0	7
GLU	31	62	3.10	3	0	7	0	1	6	0	4	5	7	3	3	0	3	7	4	0	6	1	2	0	7
PHE	25	50	2.50	2	0	2	1	6	13	0	3	4	1	1	2	3	1	3	1	4	2	0	1	0	13
GLY	48	96	4.80	8	0	4	6	13	10	5	7	3	7	1	2	5	2	6	5	3	7	0	2	0	13
HIS	14	28	1.40	2	0	0	0	0	5	0	2	1	3	0	2	0	3	1	4	0	3	0	2	0	5
ILE	37	74	3.70	3	0	4	4	3	7	2	4	6	6	2	3	5	1	5	7	4	5	2	1	0	7
LYS	33	66	3.30	9	3	4	5	4	3	1	6	2	4	2	2	5	2	2	1	4	4	0	3	0	9
LEU	31	62	3.10	4	0	5	7	1	7	3	6	4	2	2	4	0	2	3	5	2	2	0	3	0	7
MET	13	26	1.30	1	0	0	3	1	1	0	2	2	2	0	1	0	0	2	5	3	2	0	1	0	5
ASN	21	42	2.10	6	1	0	3	2	2	2	3	2	4	1	0	4	0	3	2	0	2	1	4	0	6
PRO	21	42	2.10	4	2	4	0	3	5	0	5	5	0	0	4	0	1	1	0	5	3	0	0	0	5
GLN	13	26	1.30	2	0	1	3	1	2	3	1	2	2	0	0	1	0	3	1	2	2	0	0	0	3
ARG	30	60	3.00	2	0	5	7	3	6	1	5	2	3	2	3	1	3	4	1	5	4	0	3	0	7
SER	30	60	3.00	8	0	2	4	1	5	4	7	1	5	5	2	0	1	1	0	3	5	2	4	0	8
THR	26	51	2.50	3	0	3	0	4	3	0	4	4	2	3	0	5	2	5	3	2	4	0	4	0	5
VAL	34	68	3.40	4	2	7	6	2	7	3	5	4	2	2	2	3	2	4	5	4	2	0	2	0	7
TRP	3	6	.30	0	0	0	1	0	0	0	2	0	0	0	1	0	0	0	2	0	0	0	0	0	2
TYR	18	36	1.80	3	0	1	2	1	2	2	1	3	3	1	4	0	0	3	4	4	2	0	0	0	4

TOTAL 500

Unmatched AMINO ACIDS : 0

TABLE C-20

No.	NAME	Res	SERINE Freq	Nbr	RV	Observed
1.	BOVINE GLUTAMATE DEHYDROGENASE	500	30	60	3	0
2.	NEUROSPORA GLUTAMATE ,,	453	29	58	2.9	1
3.	HORSE LIVER ALCOHOL ,,	365	25	50	2.5	0
4.	GLYCERALDEHYDE 3-PHOSPHATE-FROM PIG MUSCLE	332	19	38	1.9	1
5.	GLYCERALDEHYDE 3-PHOSPHATE-DEHYDROGENASE FROM LOBSTER MUSCLE	334	26	52	2.6	3
6.	GLYCERALDEHYDE 3-PHOSPHATE (B.STEAROTHERMOPHILUS)	334	16	32	1.6	1
7.	LACTATE DEHYDROGENASE-DOGFISH	330	26	52	2.6	0
8.	TRIOSE PHOSPHATE ISOMERASE FROM RABBIT MUSCLE	248	12	24	1.2	0
9.	TRIOSE PHOSPHATE ISOMERASE FROM CHICKEN MUSCLE	249	12	24	1.2	0
10.	TRIOSE PHOSPHATE ISOMERASE FROM COELACANTH MUSCLE	248	15	30	1.5	0
11.	ALDOLASE RABBIT MUSCLE (α)	361	21	42	2.1	0
12.	ALDOLASE RABBIT MUSCLE (α)	364	20	40	2.0	1
13.	ASPARTATE AMINOTRANSFERASE	412	27	54	2.7	2
14.	ASPARAGINASE FROM E.COLI	321	15	30	1.5	1

Contd.

Contd.

15.ACYL CARRIER PROTEIN (E.COLI)	77	3	5	0.25	0
16.ASPARTATE TRANSCARBAMYLASE	152	11	22	1.1	0
17.TRYPTOPHAN SYNTHETASE α-CHAIN-E.COLI	267	11	21	1.05	0
18.TRYPTOPHAN SYNTHETASE OF A. AEROGENES, α-CHAIN	269	12	24	1.2	0
19.TRYPTOPHAN SYNTHETASE OF S. TYPHIMURIUM, α-CHAIN	268	60	32	1.6	1
20.DIHYDROFOLATE REDUCTASE (D.R.) FROM S. FAECIUM	167	7	14	0.7	0
21.D.R. FROM E.COLI MUTANT	159	9	18	0.9	1
22.D.R. FROM E.COLI	159	9	18	0.9	1
23.D.R. (MOUSE)	186	11	22	1.1	2
24.CARBONIC ANHYDRASE B	261	25	50	2.5	3
25.CARBONIC ANHYDRASE C	260	18	35	1.75	1
26.CARBONIC ANHYDRASE C	260	17	34	1.7	1
27.CARBONIC ANHYDRASE C-SHEEP	261	18	36	1.8	3
28.CARBONIC ANHYDRASE II-RABBIT	260	20	40	2.0	3
29.λPHASE ENDOLYSIN	157	9	18	0.9	0
30. Δ^5-3-KETOSTEROID ISOMERASE	125	5	10	0.5	0
31.PENICILLINASE FROM S. AUREUS	257	19	38	1.9	0
32.PENICILLINASE	265	11	22	1.1	1
33.HUMAN ADENYLATE KINASE	195	11	22	1.1	1
34.PORCINE ADENYLATE KINASE	195	11	22	1.1	1
35.RIBITOL DEHYDROGENASE	249	13	26	1.3	1

Contd...

36.BOVINE ERYTHROCYTE SUPEROXIDE DISMUTASE	152	8	16	0.8	0
37.PHOSPHOLIPASE-A (BEE VENOM)	129	10	20	1.0	1
38.PHOSPHOLIPASE A (PIG PANCREAS)	130	12	24	1.2	2
39.PHOSPHOLIPASE A (RINGHALS)	119	6	12	0.6	0
40.PHOSPHOLIPASE A (DE-I)-COBRA	118	3	6	0.3	0
41.PHOSPHOLIPASE A (DE-II)-COBRA	119	6	12	0.6	0
42.PHOSPHOLIPASE A (DE-III)-COBRA	119	6	12	0.6	0
43.PHOSPHOLIPASE A-BITIS GABONICA	118	6	12	0.6	0
44.PHOSPHOLIPASE A_2-BOVINE	123	10	20	1.0	1
45.PHOSPHOLIPASE A_2-HORSE	125	11	22	1.1	1
46.BOVINE TRYPSINOGEN	229	34	68	3.4	2
47.PORCINE TRYPSIN	228	26	52	2.6	4
48.DOGFISH TRYPSIN	222	17	34	1.7	1
49.TRYPSIN (STREPTOMYCES GRISEUS)	221	14	28	1.4	0
50.BOVINE CHYMOTRYPSINOGEN A	245	28	56	2.8	3
51.BOVINE CHYMOTRYPSINOGEN B	245	21	42	2.1	2
52.THERMOLYSIN	316	26	52	2.6	1
53.PORCINE PEPSIN	327	43	86	4.3	6
54.PAPAIN	212	13	26	1.3	1
55.SUBTILISIN BPN'	275	37	74	3.7	9
56.SUBTILISIN CARLSBERG	274	32	64	3.2	4
57.SUBTILISIN AMYLOSACCHARITICUS	275	41	82	4.1	1 1
58.PORCINE ELASTASE	240	22	44	2.2	7
59. α-LYTIC PROTEASE	198	20	40	2.0	2

Contd.

Contd.

60.PRETHROMBIN 2 AND α -THROMBIN HUMAN	308	19	38	1.9	0
61.STREPTOCOCCAL PROTEINASE	253	21	42	2.1	3
62.PROTEASE A FROM STREPTOMYCES GRISEUS	182	22	44	2.2	1
63.PROTEASE B FROM STREPTOMYCES GRISEUS	184	22	44	2.2	3
64.CARBOXYPEPTIDASE A	307	32	64	3.2	8
65.BOVINE CARBOXYPEPTIDASE B	306	27	54	2.7	2
66.DNA-DEPENDENT RNA POLYMERASE(α -SUBUNIT)-E.COLI	329	17	34	1.7	2
67.RHODANESE-BOVINE LIVER	293	21	42	2.1	0
68.BOVINE RIBONUCLEASE	124	15	30	1.5	5
69.PORCINE RIBONUCLEASE	124	13	26	1.3	11
70.HORSE RIBONUCLEASE	125	14	28	1.4	7
71.OVINE RIBONUCLEASE	124	16	32	1.6	7
72.GOAT RIBONUCLEASE	124	17	34	1.7	7
73.RAT RIBONUCLEASE	127	15	30	1.5	7
74.MUSKRAT RIBONUCLEASE	124	15	30	1.5	8
75.CHINCHILLA RIBONUCLEASE	124	14	28	1.4	2
76.GUINEA PIG RIBONUCLEASE A	124	18	36	1.8	8
77.GUINEA PIG RIBONUCLEASE B	128	17	34	1.7	6
78.GIRAFFE RIBONUCLEASE	124	16	32	1.6	6
79.RED-DEER RIBONUCLEASE	124	15	30	1.5	5
80.ROE-DEER RIBONUCLEASE	124	15	30	1.5	5
81.REINDEER RIBONUCLEASE	124	15	30	1.5	6

contd..

Contd.

82.MOOSE RIBONUCLEASE	124	15	30	1.5	5
83.FALLOW DEER RIBONUCLEASE	124	15	30	1.5	5
84.DROMEDARY RIBONUCLEASE	124	18	36	1.7	2
85.COYPU RIBONUCLEASE	128	16	32	1.7	9
86.ELAND RIBONUCLEASE	124	16	32	1.6	7
87.GNU RIBONUCLEASE	124	16	32	1.6	7
88.TOPI RIBONUCLEASE	124	17	34	1.7	7
89.PIKE-WHALE RIBONUCLEASE	124	11	22	1.1	1
90.RIBONUCLEASE OF BACILLUS AMYLOLIQUEFACIENS	110	9	18	0.9	1
91.RIBONUCLEASE T1	104	15	30	1.5	6
92.RIBONUCLEASE St	102	3	6	0.3	0
93.RIBONUCLEASE U2	113	10	20	0.9	1
94.STAPHYLOCOCCAL NUCLEASE STRAIN V8	149	5	10	0.5	0
95.STAPHYLOCOCCAL NUCLEASE FOGGI STRAIN	149	5	10	0.5	0
96.BOVINE PANCREATIC DEOXY-RIBONUCLEASE A	257	30	60	3.0	4
97.HUMAN LEUKAEMIA LYSOZYME	129	6	12	0.6	0
98.HUMAN MILK LYSOZYME	130	6	12	0.6	0
99.LYSOZYME FROM BABOON MILK	130	7	14	0.7	0
100.HEN EGG LYSOZYME	129	10	20	1.0	1
101.GUINEA-HEN EGG LYSOZYME	129	10	20	1.0	1
102.DUCK EGG LYSOZYME II	129	11	22	1.1	0
103.DUCK EGG LYSOZYME III	129	9	18	0.9	0

Contd....

Contd.

104.T4 PHAGE LYSOZYME	164	6	12	0.6	0
105.LYSOZYME OF CHALAROPSIS	211	22	44	2.2	3
106.HUMAN α-LACTALBUMIN	123	8	16	0.8	1
107.GUINEA-PIG α-LACTALBUMIN	123	8	16	0.8	1
108.BOVINE α-LACTALBUMIN	123	7	14	0.7	1
109.KAZAL'S INHIBITOR	56	2	4	0.2	0
110.COW COLOSTRUM TRYPSIN INHIBITOR (COMPONENT B2)	67	3	6	0.25	0
111.KALLIKREIN INACTIVATOR (TRASYLOL)	58	1	2	0.1	0
112.TRYPSIN INHIBITOR H FROM PORCINE PANCREAS	52	5	10	0.5	0
113.TRYPSIN INHIBITOR I FROM PORCINE PANCREAS	56	6	12	0.5	0
114.PROTEINASE INHIBITOR FROM CANINE SUBMANDIBULAR GLANDS	117	8	16	0.8	0
115.STREPTOMYCES SUBTILISIN INHIBITOR	113	9	18	0.9	1
116.ISOINHIBITOR K FROM SNAIL	58	4	8	0.4	0
117.INHIBITOR II FROM RUSSELL'S VIPER VENOM	60	2	4	0.2	0
118. PROTEINASE INHIBITOR II-RINGHAL'S COBRA	57	1	2	0.1	0
119.PROTEINASE INHIBITOR II-NAJA NIVEA	57	1	2	0.1	0
120.CHYMOTRYPTIC INHIBITOR I FROM POTATOES	71	5	10	0.5	0
121.CHYMOTRYPTIC INHIBITOR I FROM POTATOES (SUBUNIT A)	84	4	8	0.4	0

Contd....

Contd.

122. ACTIVE FRAGMENT OF POTATO PROTEINASE INHIBITOR IIA	45	4	8	0.35	0
123.CARBOXYPEPTIDASE INHIBITOR	39	2	4	0.2	0
124.SOYABEAN PROTEINASE INHIBITOR C-II	76	12	24	1.15	5
125.SOYABEAN PROTEINASE INHIBITOR D-II	75	12	24	1.15	4
126.SOYABEAN TRYPSIN INHIBITOR (KUNITZ)	181	11	22	1.1	0
127.TRYPSIN INHIBITOR D-II-SOYABEAN	75	12	24	1.15	4
128.BOWMAN-BIRK SOYABEAN TRYPSIN INHIBITOR	71	9	18	0.9	1
129.ARACHIS HYPOGAEA TRYPSIN INHIBITOR	48	3	6	0.3	0
130.LIMA BEAN PROTEASE INHIBITOR COMPONENT IV	84	15	30	1.4	1
131.TRYPSIN INHIBITOR II FROM GARDEN BEAN	71	11	22	1.1	1
132.PROTEASE INHIBITORS FROM PINEAPPLE STEM A-CHAIN	41	2	4	0.2	0
133.SOMATOMEDIN B	44	3	6	0.3	0
134.HUMAN HAEMOGLOBIN α-CHAIN	141	11	22	1.1	0
135.MONKEY HAEMOGLOBIN α-CHAIN	141	12	24	1.2	0
136.HAEMOGLOBIN α-CHAIN-CAPUCHIN MONKEY	141	12	24	1.2	0
137.HAEMOGLOBIN α-CHAIN-SAVANNAH MONKEY	141	12	24	1.2	0
138.HAEMOGLOBIN α-CHAIN-JAPANESE MONKEY	141	12	24	1.2	0

Contd.

139.HAEMOGLOBIN α-CHAIN-HANUMAN LANGUR	141	11	22	1.1	0
140.HAEMOGLOBIN α-CHAIN- ORANGUTAN	141	11	22	1.1	0
141.HAEMOGLOBIN α-CHAIN- SLENDER LORIS	141	13	26	1.3	0
142.HAEMOGLOBIN α-CHAIN- SLOW LORIS	141	14	28	1.4	0
143.HORSE HAEMOGLOBIN α-CHAIN	141	13	26	1.3	1
144.KANGAROO HAEMOGLOBIN α-CHAIN	141	10	20	1.0	0
145.HAEMOGLOBIN α-CHAIN- ECHIDNA (MAJOR HAEMOGLOBIN HB-IB)	141	12	24	1.2	0
146.HAEMOGLOBIN α-CHAIN-ECHIDNA (MAJOR HAEMOGLOBIN HB-IA)	141	11	22	1.1	0
147.HAEMOGLOBIN α-CHAIN-ECHIDNA (MINOR HAEMOGLOBIN HB-IIA)	141	11	22	1.1	0
148.HAEMOGLOBIN α-CHAIN-OPOSSUM	141	13	26	1.3	1
149.RABBIT HAEMOGLOBIN α-CHAIN	141	12	24	1.2	0
150.HAEMOGLOBIN α-CHAIN-PIG	141	10	20	1.0	0

(24-28; TABLE C.20), involved in hydration of CO_2, b) the proteases, trypsin (47), pepsin (53), Subtilisin(55-57), Carboxypeptidase (64), c) trypsin inhibitors (124-127) d) elastase, involved in destruction of elastin and responsible for the delicate elastin -α_1-antitrypsin balance (58), e) ribo nuclease (68-93) and f) de-Oxy ribo nuclease (96). Thus nucleases and proteases harbor excessive Ser-Ser combination. The presence of 5 serine residues in a row in certain ribo nucleases (74, 84) and 4 Ser in a row in subtilisin (58) must be considered significant, particularly in view of the relative rarity of even 2 Ser. in a row in most proteins. Whilst the implications of this may be complex, it tends to support the earlier conjecture that protein evolution was based on a synthon approach, selecting segments appropriate for the needed function.

Complementing the above notion, it is possible that the rarity of Ser-Ser bond in proteins could arise via potential instability, leading to Gly-Ser/ Ser-Gly via retro aldol reaction in cases where the Ser-Ser unit is either unprotected or not complexed.

Above all an analysis such as presented in TABLE C.20 could generate several experimental leads which would have relevance to protein evolution.

The enzyme Cytochromone is present in species separated by aeons in the evolutionary scale. Indeed the analysis cited span from E. Coli Cytochrome to human Cytochrome. An appropriate querry would be whether preferences for neighbours are retained in the evolution of the species. An affirmative response would be interesting particularly with reference to very early stages in protein evolution. Preferences for neighbours of value 3 and above have been processed from TABLE C.2 - TABLE C.18 and presented in TABLE C.21 Cytochrome family is rich in lysine residues and interestingly the overwhelming preference exhibited by these lysine residues to have another lysine as

TABLE C-21

CYTOCHROMES

	b E.Coli	b Yeast	c Buck wheat	c Wheat germ	c Blow fly	c Locus	c Insect	c Moth	c Silk worm	c Guanco	H/c Rabbit	H/c Camel	H/c Horse	H/c Bovine	LiverB5 Chicken	LiverB5 Monkey	LiverB5 Human
ALA	Ala	Pro	Glu	Gly	Ala	Gly	Gly	Asn	Asn	-	-	-	-	-	-	-	-
CYS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ASP	Asp	-	-	Asp	-	-	-	-	-	-	-	-	-	-	-	-	-
GLU	Ala	-	-	-	-	Asn	Asn	-	Asn	-	-	-	Lys	-	-	Glu	Glu
PHE	-	-	-	-	-	-	-	Gly	-	-	-	-	-	-	-	-	-
GLY	-	Pro	Pro	Ala	Lys	Glu/ Lys	Lys	Ala Lys Phe Pro	Ala Lys Pro	Lys	Lys	Lys	Lys	Lys	Gly	Gly	Gly
HIS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ILE	-	Val	-	-	-	-	-	-	-	-	-	-	-	-	Ile	-	-
LYS	Val	Gln	Asn	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	-	Ser	Ser
LEU	Lys	Pro	Asn	Tyr	-	Lys	Lys	-	-	-	-	-	-	-	-	Glu	-
MET	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ASN	Ala	-	Lys	Pro	Pro	Glu/ Pro	Asn/ Pro	Ala	Ala	Lys	Lys	Lys	Lys	Lys	-	-	-
PRO	-	Pro/ Ala	Gly	Gly	Asn	Asn	Asn	Gly	Gly	-	-	Gly	Gly	-	-	-	-
GLN	Ala	Lys	-	-	-	-	-	Ala	-	-	-	-	-	-	-	-	-
ARG	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SER	-	-	Tyr	Tyr	-	-	-	-	-	-	-	-	-	-	-	Lys	Lys
THR	-	-	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Gly	Gly/ Lys	Lys	Lys	Gly/	-	-	-
VAL	Lys	Ile	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TRP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TYR	-	-	Leu/ Ser	Leu/ Ser	-	-	-	-	-	-	-	-	-	-	-	-	-

neighbour is largely retained through the evolutionary span ! in the cases cited in TABLE C.21, although retention of preferences are seen, they are nowhere near as convincing as this lysine example.

The analysis of available sequences described above complements experimental observations and has led to diverse avenues for pertinent experimental scrutiny.

PEPTIDE BOND FORMATION AT THE MICELLAR INTERFACE

The selective environments described thus far, leading to peptide bond formation, had as the focus, polar interactions wherein, polar side chains of coded α-amino acids play a pivotal role. The prepondarance of hydrophobic residues in proteins clearly points to circumstances conducive for peptide bond formation involving hydrophobic side chains.

Hydrophobic bonding, involving the accumulation of hydrophobic side chains of coded α-amino acids, is a key feature in the folding of enzymes leading to unique structures.

It was envisaged that, in the event, amino acids having hydrophobic side chains could be aligned with an appropriate condensing agent on a micellar support, peptide bond formation will take place at the water interface leading to preferential formation of peptides involving hydrophobic amino acid residues.

Amongst the reverse micellar systems harboring water pools that have been studied extensively in recent years, the most appropriate one was considered, as the one formed by addition of bis-2-ethylhexyl sodium sulfosuccinate (AOT) in isooctane containing water. Studies using this surfactant have shown that 100 mM amount of the compound in isooctane can harbor as much as

2% water, giving rise to clear solutions. Extensive studies using this system have shown that reverse micelle formedcould be represented as shown in CHART C.17. The strategy called for the condensing reagent in the role of the co-surfactant. Consequently, the compound to be used, would require hydrophobic side chains compatable with that of AOT. Consequently, dioctadecylcarbodiimide (DODCI) possessing the polar carbodiimide unit attached to two hydrophobic tails, seemed most appropriate. The desired compound (DODCI) was prepared from octadecylamine, which on reaction with CS_2 gave dioctadecylthiourea (40). Compound (40) was readily transformed to DODCI (39) via oxidation with yellow HgO. The overall yield of DODCI was 80% from octadecylamine (CHART C.18).

The structural assignment is fully supported by spectral and analytical data.

Dioctadecylcarbodiimide (DODCI) (39) :

mp. 49-50°C

ir : ν_{max}(KBr) cm^{-1} : 2130 (carbodiimide).

nmr : $\delta(CDCl_3)$: 0.7-2.5 (m, 70 H), 3.3, 3.7 (t, t, 4H, $NC\underline{H}_2CH_2$).

ms : m/z : 546 (M^+).

Dioctadecylthiourea (40) :

mp. 96-97°C.

ir : ν_{max}(KBr) cm^{-1} : 3280 (-NH), 1590, 1490, 1375 (thiourea).

ms : m/z : 580 (M^+-S).

CHART C-17

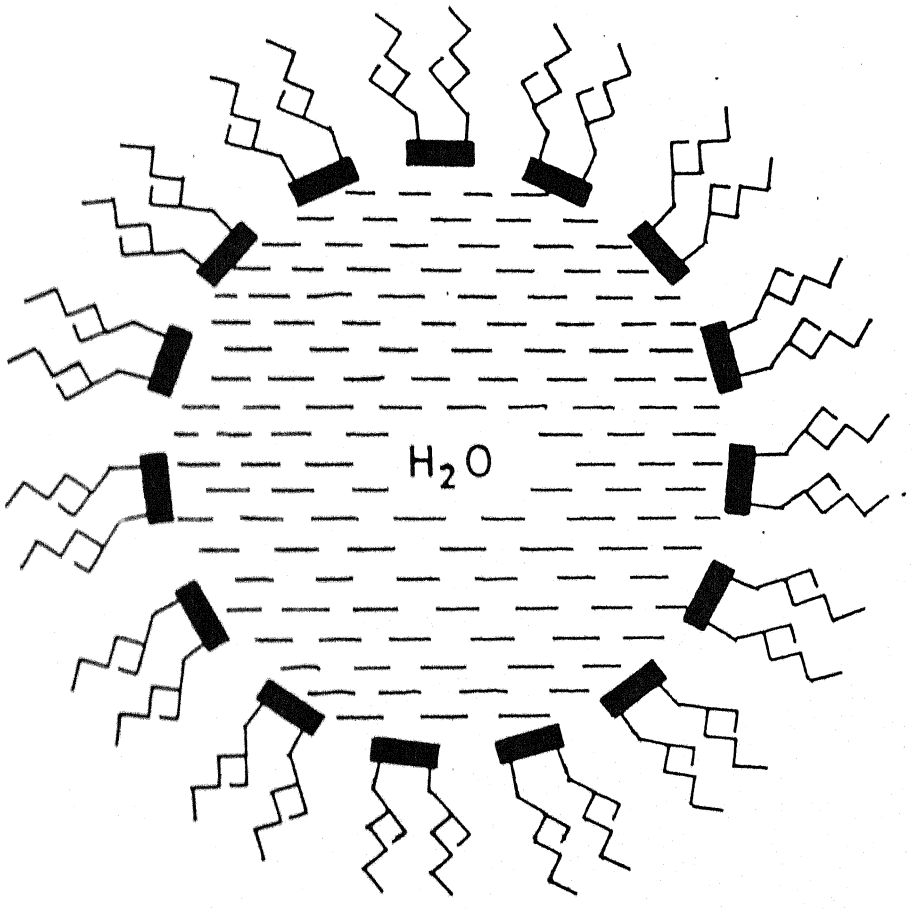
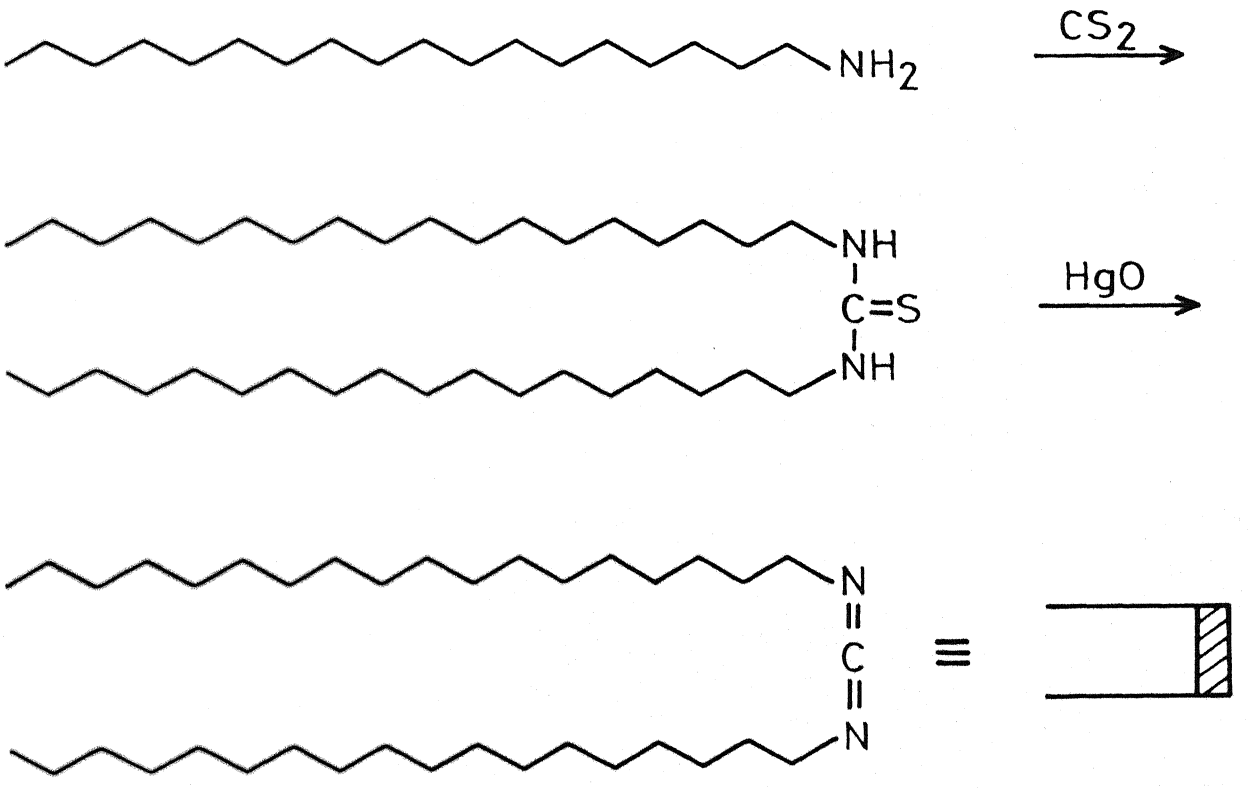


CHART C-18



DODCI mp 50°C

As expected, DODCI is easily soluble in hydrocarbon solvents. At the outset, it was considered desirable to establish the efficacy of DODCI as a condensing agent. Equimolar amounts of DODCI in dioxane, effectively, brought about the condensation of BzPhe and cyclohexylamine, BzLeu and cyclohexylamine, BzLeu and Gly-OMe and BzMet and Gly-OMe leading to the formation of, respectively, (41) (55%), (42) (73%), Bz-Leu-Gly-OMe (43) (64%) and BzMet-Gly-OMe (44) (40%).

$BzPheCONHC_6H_{11}$ (41) :

mp. 197-198°C.

ir : ν_{max}(KBr) cm^{-1} : 3310 (-NH), 1650, 1540 (amide).

nmr : $\delta(CDCl_3)$: 1.0-2.2 (m, 10H, cyclohexyl), 3.2 (m, 3H, CH-CH_2-Ph + CONH-CH), 4.8 (m, 1H, tert.proton), 7.2 (s, 5H, Ph), 7-7.9 (m, 5H, aromatic).

ms : m/z : 350 (M^+), 229 (M^+-BzNH), 224 (M^+-CONH cyclohexyl).

$BzLeuCONHC_6H_{11}$ (42) :

mp. 179-181°C.

ir : ν_{max}(KBr) cm^{-1} : 3300 (-NH), 1620, 1540 (amide).

nmr : $\delta(CDCl_3)$: 0.9 (d, 6H, $CH(CH_3)_2$), 1-2.2 (m, 13H, cyclohexyl + CH_2-$CH(CH_3)_2$), 4.5 (m, 1H, tert.proton), 7.2-7.9 (m, 5H, aromatic).

BzLeu-Gly-OMe (43) :

mp. 178°C.

ir : ν_{max}(KBr) cm^{-1} : 3300 (-NH), 1745 (ester), 1640,1450 (amide).

nmr : δ ($CDCl_3$) : 1.0 (d, 6H, CH-($\underline{CH_3}$)$_2$), 1.4 (m, 1H, $\underline{CH}$-($CH_3\underline{CH_3}$), 1.7 (m, 2H, CH-$\underline{CH_2}$-$CHMe_2$), 3.7 (s, 3H, COO$\underline{CH_3}$), 4.1 (d, 2H, NH-$\underline{CH_2}$-CO_2Me), 4.8 (m, 1H, tert.proton), 7-7.9 (m, 7H, aromatic + 2 x NH).

ms : m/z : 307 (M^+), 250 (M^+-CH_2CHMe_2).

Bz-Met-Gly-OMe (44) :

mp. 134-5°C.

ir : ν_{max}(KBr) cm^{-1}, 3290 (-NH), 1750 (ester), 1560, 1445 (amide).

nmr : δ ($CDCl_3$) : 2.2 (s, 3H, S-$\underline{CH_3}$), 2.1-2.5 (m, 2H, CH-$\underline{CH_2}$-CH_2-S-CH_3), 2.8 (t, 2H, CH-CH_2-$\underline{CH_2}$-S-CH_3), 3.2 (t, 1H, $\underline{NH}$-CH_2), 3.75 (s, 3H, COO$\underline{CH_3}$), 4.1 (br, 2H, NHCH$\underline{CH_2}$), 4.2 (br, 1H, PhCO$\underline{NH}$), 5.0 (m, 1H, tert.proton), 7.2-8.1 (m, 7H, aromatic + NH).

ms : m/z : 325 MH^+, 250 ($M+H^+$-CH_2CH_2S-CH_3).

The results of the above study are compared with yields obtained using the traditional DCC-HOBt method. The results are presented in CHART C.19, which would show that in several cases, the use of DODCI gave better yields. In addition, the work up procedures are easier with DODCI.

CHART C.19

DODCI IS MORE CONVENIENT THAN DCC IN REGULAR PEPTIDE SYNTHESIS SINCE DI-OCTADECYLUREA (DODU) IS INSOLUBLE

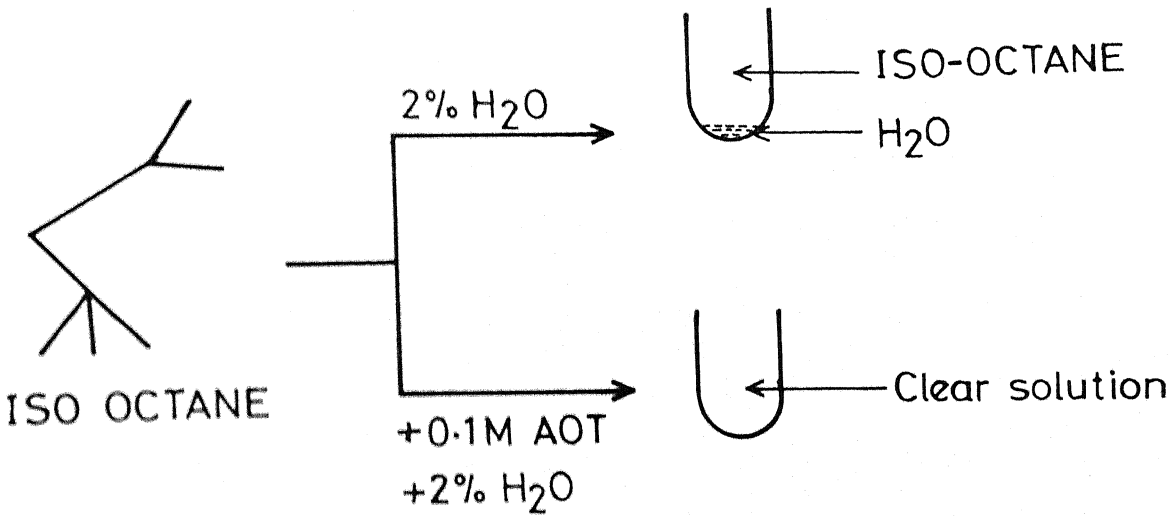
PRODUCT	YIELD(%) DODCI	DCC-HOBT
Bz-LEU-GLY-OMe	64	55
Bz-MET-GLY-OMe	40	70
Bz-LEU-CONH-C_6H_{11}	73	59
Bz-PHE-CONH-C_6H_{11}	55	67

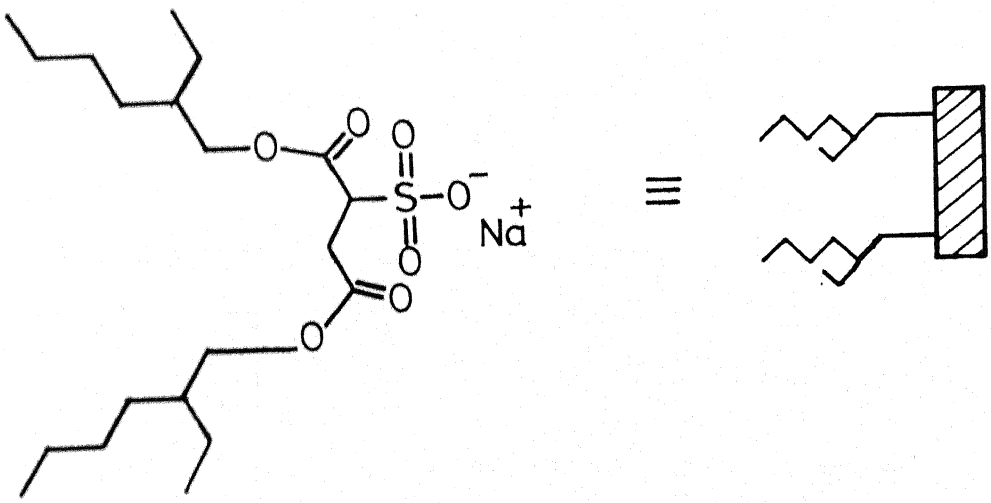
As shown in CHART C.20, addition of water (2%) to isooctane leads to the clear separation of two layers. In sharp contrast, isooctane to which 0.1 mole of AOT has been added, provides clear solution, on addition of 2% water giving rise to reverse micellar system as illustrated in CHART C.17. The polar head of AOT derives significant contribution from the sulphonate salt as well as the succinate ester oxygen.

Significant hydrophobic surface is created by the branched ester function as shown in CHART C.20. CHART C.21 offers a probable profile of the reverse micelle form in isooctane to which DODCI has been added. As could be seen from this DODCI (39) can act as a co-surfactant and elegantly align along the micellar surface with the carbodiimide functionality facing the water cavity. Two possible situations can arise when coded α -amino acids are added to the DODCI - AOT reverse micelle composite. In the case of α -amino acids possessing hydrophobic residues, the side chains can be expected to be a part of the micellar surface with α -amino acid moiety facing the water-pool. Such an arrangement, illustrated in CHART C.22a, would lead to the activated ester followed by peptide bond formation. On the other hand, in the case of amino acids carrying polar side chains, they could be expected to be present mostly in water pool and the peptide bond formation involving these compounds would be governed by their interaction with DODCI anchored at the micellar interface CHART C.22b.

Based on models presented in CHART C.22a and CHART C.22b, it would be anticipated that the peptide bond involving hydrophobic residues would be more easily formed in isooctane harboring micelles incorporating AOT and DODCI compared to the polar α-amino acids. This expectation has been very satisfactorily realized.

CHART C-20


ISO-OCTANE
H_2O
2% H_2O
ISO OCTANE
Clear solution
+0·1M AOT
+2% H_2O


O
O
S
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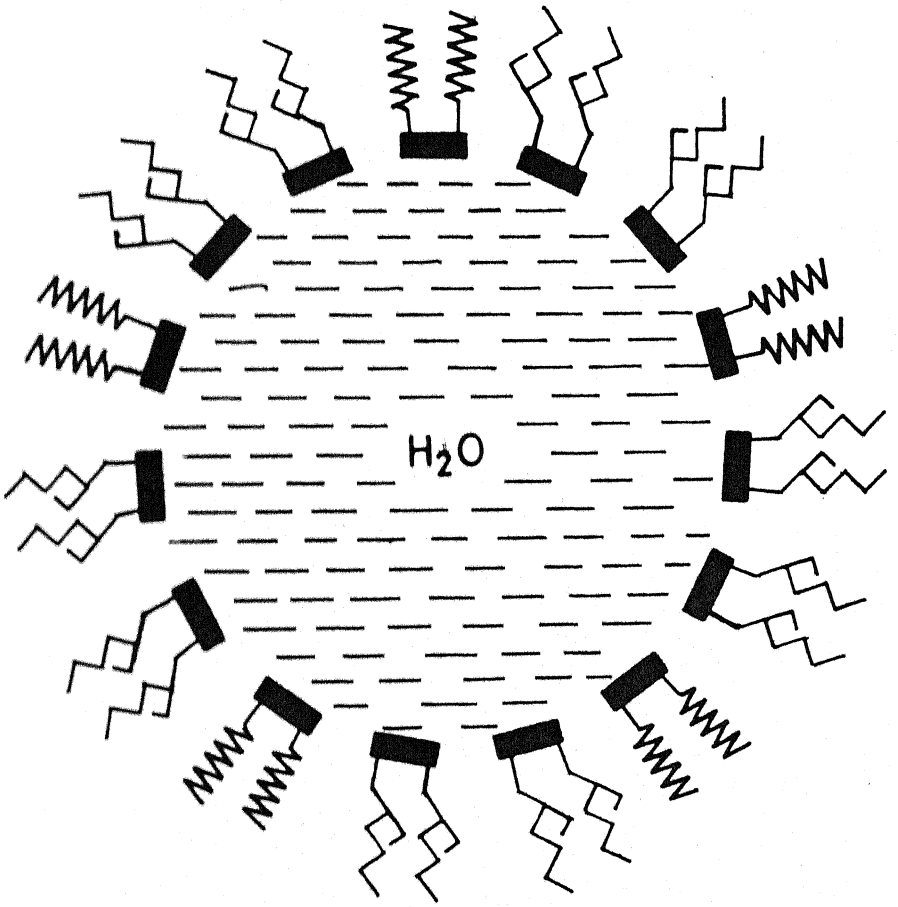
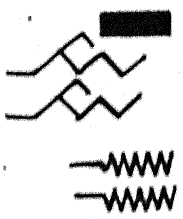
Na^+
≡

CHART C-21

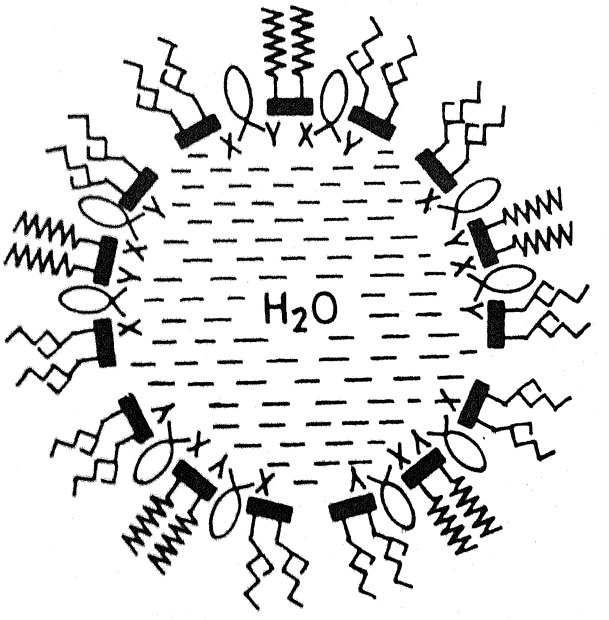




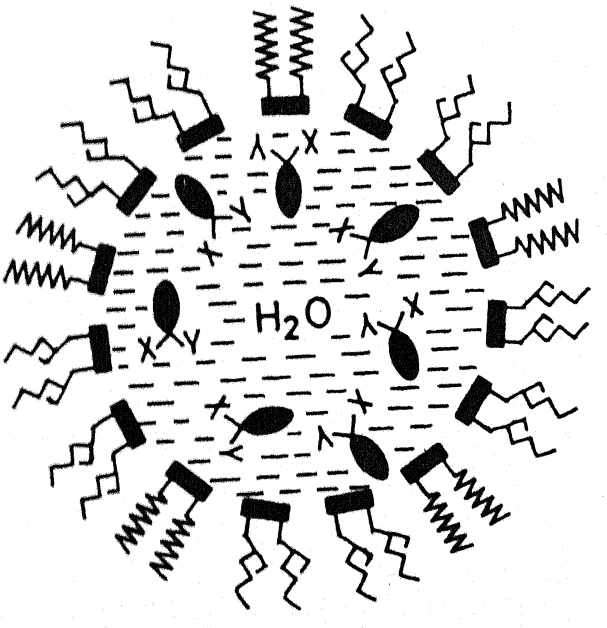
Polar head of AOT & DODCI

Hydrophobic tails of AOT

Hydrophobic tails of DODCI



(a)



(b)

Polar head of AOT & DODCI

Hydrophobic tails of AOT

Hydrophobic tails of DODCI

$x = NH_2$; $Y = COOH$; Hydrophobic residue;

Polar residue.

In a typical experiment, 0.5 mmol of N- protected amino acid was mixed with 10 ml of stock solution (prepared by adding 10 m mol of AOT to 100 ml of isooctane containing 2 ml of water) and 0.5 mmol of DODCI followed by 0.5 mmol of amino acid methyl ester which was usually freshly prepared. The results of this study are presented in CHART C.23. As could be seen from CHART C.23, the yields of dipeptides BzLeu-Leu-OMe (30), BzPhe-Phe-OMe (46), Bz-Phe-Pro-OMe (47), BzPhe-Leu-OMe (48) and BzTrp-Trp-OMe (49) possessing hydrophobic side chains were significantly higher compared to BzGlu-Glu-di-OMe (33) and Bz Asp-Asp-di-OMe (50) possessing polar side chains.

BzPhe-Phe-OMe (46) :

mp. 178-179°C.

ir : ν_{max}(KBr) cm^{-1} : 3320 (-NH), 1745 (ester), 1665, 1640, 1550, 1530 (amide).

BzPhe-Pro-OMe (47) :

Thick syrup

ir : ν_{max}(neat) cm^{-1} : 3340 (-NH), 1750 (ester), 1640, 1545 (amide).

nmr : $\delta(CDCl_3)$: 2.0 (m, 4H, $(CH_2)_2$), 3.23 (d, 2H, -CHCH_2Ph), 3.66 (s, 3H, COOCH_3) 3.9 (m, 2H, -N-CH_2), 4.4 (m, 1H, -CH), 5.2 (m, 1H, -CH), 7.0 - 7.9 (m, 11 H, -NH, 2 x Ph).

BzPhe-Leu-OMe (48) :

mp. 143°C.

ir : ν_{max}(KBr) cm^{-1} : 3310 (-NH), 1760 (ester), 1640, 1550 (amide).

nmr : δ ($CDCl_3$) : 0.8 (d, 6H, $CH(C\underline{H}_3)_2$), 1.1 (m, 1H, $C\underline{H}(CH_3)_2$), 1.5 (m, 2H, $C\underline{H}_2CH(CH_3)_2$), 3.1 (d, 2H, CH-$C\underline{H}_2$-Ph), 3.6, 3.7 (s, s, 3H, $COOC\underline{H}_3$), 4.5 (br, 1H, tert.proton), 4.9 (q, 1H, tert.proton), 7.2 (s, 5H, -CH_2-Ph), 6.7 - 7.7 (m, 7H, NH, amide Ph).

BzTrp-Trp-OMe (49) :

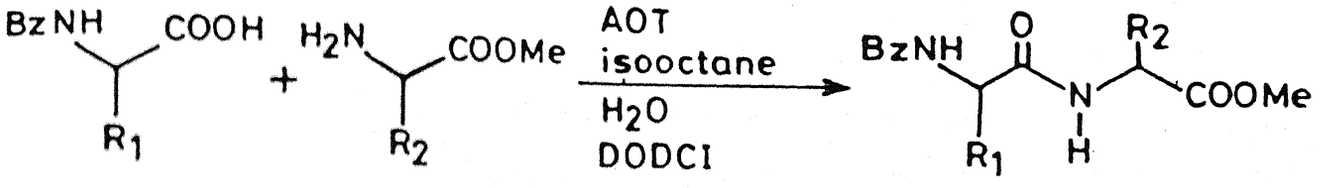
mp.188-190°C.

ir : ν_{max}(KBr) cm^{-1} : 3400, 3300 (-NH), 1740 (ester), 1640, 1610, 1520 (amide).

nmr : δ($CDCl_3$) : 2.9 (m, 4H, 2 x CH$C\underline{H}_2$-indole), 3.5 (s, 3H, $COOC\underline{H}_3$), 4.6 (m, 2H, tert.protons), 6.1-8.0 (m, 19 H, 2 x indole + 2 x NH+Ph).

As could be seen from CHART C.23, there is a correlation between relative hydrophobicity of the side chain to the isolated yield of dipeptide. This correlation is in accord with anticipation based on the profile of the system (CHART C.22). An exception to this is Phe-Pro, where the isolated yield of the dipeptide is as expected for hydrophobic residues, although, in relative hydrophobicity terms, proline is considered as one having polar side chain since

CHART C-23



R_1	ϕ	R_2	ϕ	Isolated dipeptide yield (%)
LEU	1.8	LEU	1.8	20
PHE	2.5	PHE	2.5	26
PHE	2.5	PRO	-3.3*	34
PHE	2.5	LEU	1.8	31
TRP	3.4	TRP	3.4	40
GLU	- 0.3	GLU	-0.3**	13
ASP	- 0.2	ASP	-0.2**	11

ϕ = Rel. Hydrophobicity GLY = 0 (Kcal mol^{-1})

*Exhibits unusual solubility profile

H_2O : 162g/100ml EtOH : 70g/100ml

**Since ω-Esters were used ϕ taken for GLN/ASN

(values for GLU = -9.9 ; ASP = -7.4)

the imino acid nature of this compound makes it soluble to the extent of 152 g/ 100 ml at 20°. However in terms of alignments, it can be anticipated that the hydrophobic methylene residues of this amino acid would line up at the micellar interface like a hydrophobic residue which would explain the high yield of dipeptide from this reaction.

The results obtained in the present work show, for the first time, the use of reverse micelles for the formation of peptide bond under non-enzymatic conditions[31]. Whilst a fit between that anticipated on the basis of selective peptide bond formation at the micellar interface (vide supra) and the results presented in CHART C.23 could be seen, the importance of several other factors leading to the observed results can not be ruled out. For example, the extent of dipeptide formation can be controlled by the efficiency with which the amino acids could reach the interface and align with the condensing agent for the activated ester formation leading to peptide synthesis. This process would be easier in the case of hydrophobic amino acids which would account for the higher yields of dipeptides formed. Irrespective of the variation with respect to the interpretation of the results cited in CHART C.23 the basic notion that the peptide bond formation does take place at the micellar interface seems appropriate. Having established the preference for peptide bond formation with hydrophobic amino acids, preliminary experiments were carried out to use this model for the selective peptide bond formation in a competitive environment.

The strategy that was used in the preliminary competitive experiments involved the prior formation of the activated ester at the interface involving the carboxyl residue of N- γ - C-protected Glutamic acid and N- β - C-protected Aspartic acid . To such a system was introduced half - equivalent of Leu-OMe and Glu-di-OMe / Asp-di-OMe . It was envisaged - -

that these compounds would have to diffuse through the isooctane and that the concentration of Leu-OMe would be considerably more at the interface compared to either Aspdi-OMe or Gludi-OMe. Consequently, this experiment could be expected to produce either BzGlu-Leu-OMe or BzAsp-Leu-OMe in preference to respectively, BzGlu-Glu-di-OMe and BzAsp-Aspdi-OMe. In the event, the two competitive experiments on work up followed by chromatography gave, in the first case 23% yield of BzGlu(γ -OMe)-Leu-OMe and practically none of BzGlu(γ -OMe)-Gludi-OMe. In the second case, the competitive experiment yielded 19% of BzAsp(β -OMe)-Leu-OMe (45) and 4% of BzAsp(β -OMe)-Aspdi-OMe.

BzAsp(β -OMe)-Leu-OMe (45) :

Thick syrup

ir : ν_{max}(neat) cm^{-1} : 3330, 3300 (-NH), 1750 (ester), 1650, 1555 (amide).

nmr : δ($CDCl_3$) : 1.0 (d, 6H, $HC(C\underline{H}_3)_2$), 1.25 (m, 1H, $CH_2C\underline{H}(CH_3)_2$), 1.65 (m, 2H, $CH\text{-}C\underline{H}_2CH(CH_3)_2$), 3.0 (m, 2H, $CH\text{-}C\underline{H}_2COOCH_3$), 3.7 (s, s, 6H, 2 x $COOC\underline{H}_3$), 4.6, 4.9 (m, m, 1H, 1H, tert.protons), 7.0-7.9 (m, 7H, aromatic, 2 x NH).

BzAsp(β -OMe)-Asp-(β -OMe)-OMe (50) :

mp. 136°C.

ir : ν_{max}(KBr) cm^{-1} : 3300 (-NH), 1730 (ester), 1645, 1530 (amide).

nmr : δ ($CDCl_3$) : 2.9 (m, 4H, 2 x $CHC\underline{H}_2COOMe$), 3.65, 3.75 (3H, 6H, 2 x $COOC\underline{H}_3$), 4.9 (m, 2H, tert.protons), 7.4-7.9 (m, 7H, aromatic + 2.NH).

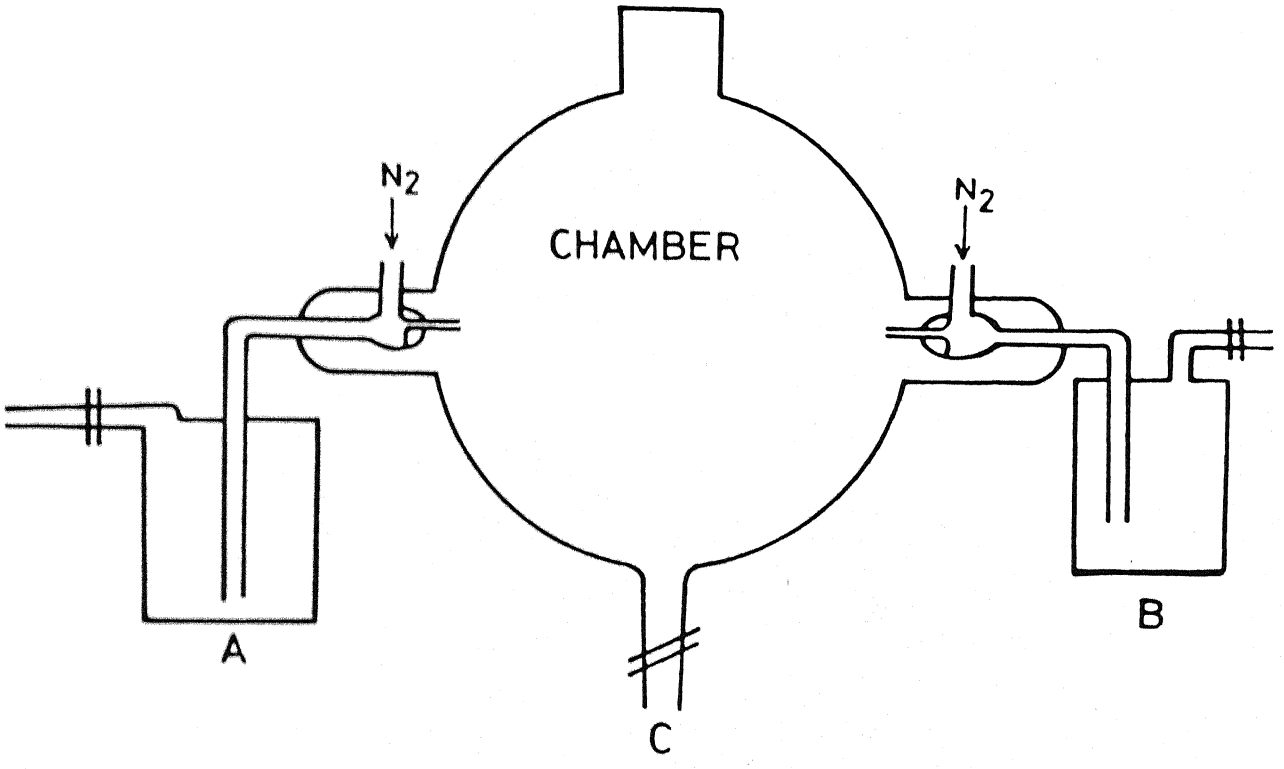
Above all, the experiments, thus far described involving reverse micellar systems incorporating AOT, generate very attractive leads for further work which would include the use of the micellar interface as a support for peptide elongation, the control of preferences in peptide bond formation and selectivity in the attachment of hydrophobic residues.

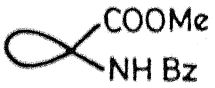
It was considered that problems related to diffusion of substrates could be minimized by generating the reverse micellar system in aerosol space. To this end the reactor illustrated in CHART C.24 was designed. It was considered that such a reactor could efficiently bring about the peptide bond formation involving a water soluble amino acid and water insoluble one. The specific experiment that was attempted, involved charging the chamber A with an equivalent amount of BzLeu-OMe in isooctane harboring the reverse micellar system described earlier. Chamber B, in turn, was charged with equivalent amounts of water soluble carbodiimide (22) and Glycine in pH 10 borate buffer. Injection of N_2 leads to discharge, from both the chambers, of contents in the form of very fine spray and it was felt that the alkaline buffer would hydrolyse some of the BzLeu-OMe in the aerosol space, permitting further condensation involving the water soluble carbodiimide leading to BzLeu-Gly, that is insoluble. The envisaged events that were anticipated to take place, are illustrated in CHART C.25. In the event, however, the yield of BzLeu-Gly-OH obtained was very poor, thus precluding a rational analysis of this experiment.

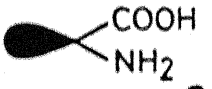
The work described thus far hopefully brings out the fact that a

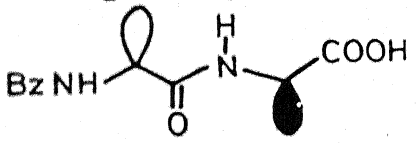
CHART C-24

PEPTIDE BOND FORMATION IN AEROSOL
NOVEL REACTOR DESIGN



A :  + AOT (0·1M) + H_2O (2%) IN ISO OCTANE

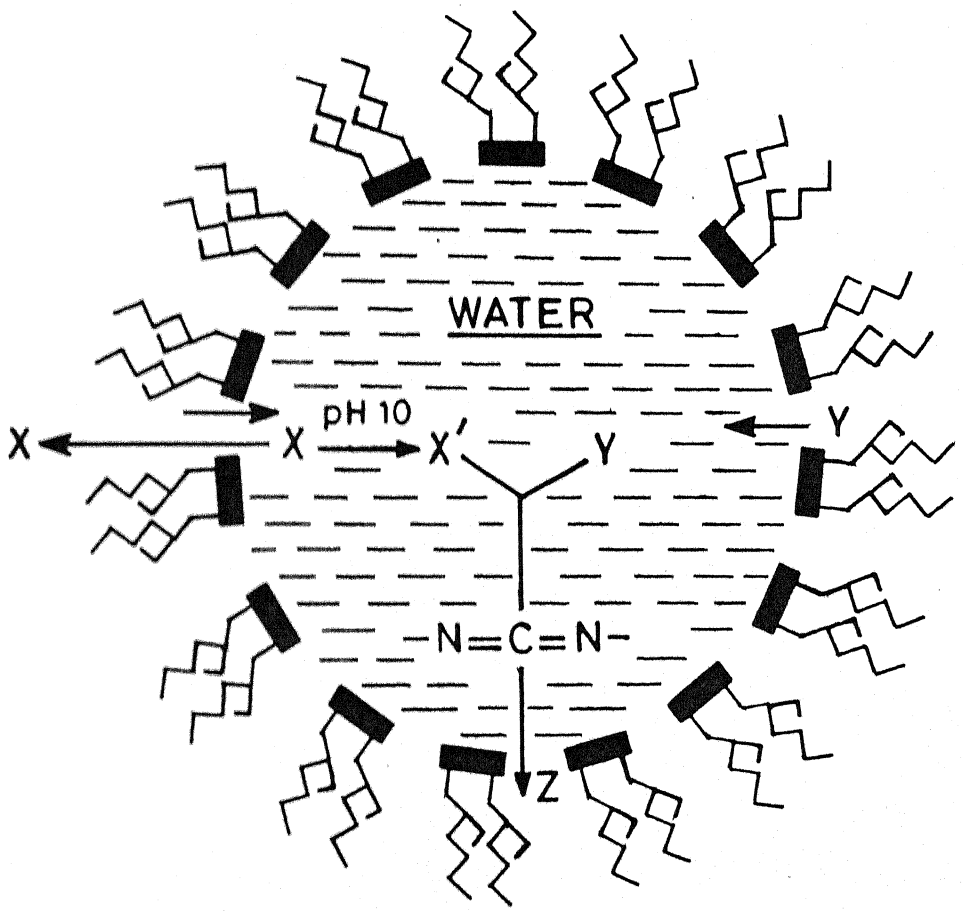
B :  + R-N=C=N-R′ + pH 10 Borate buffer (Water soluble)

C = 

 = Leu

 = Gly

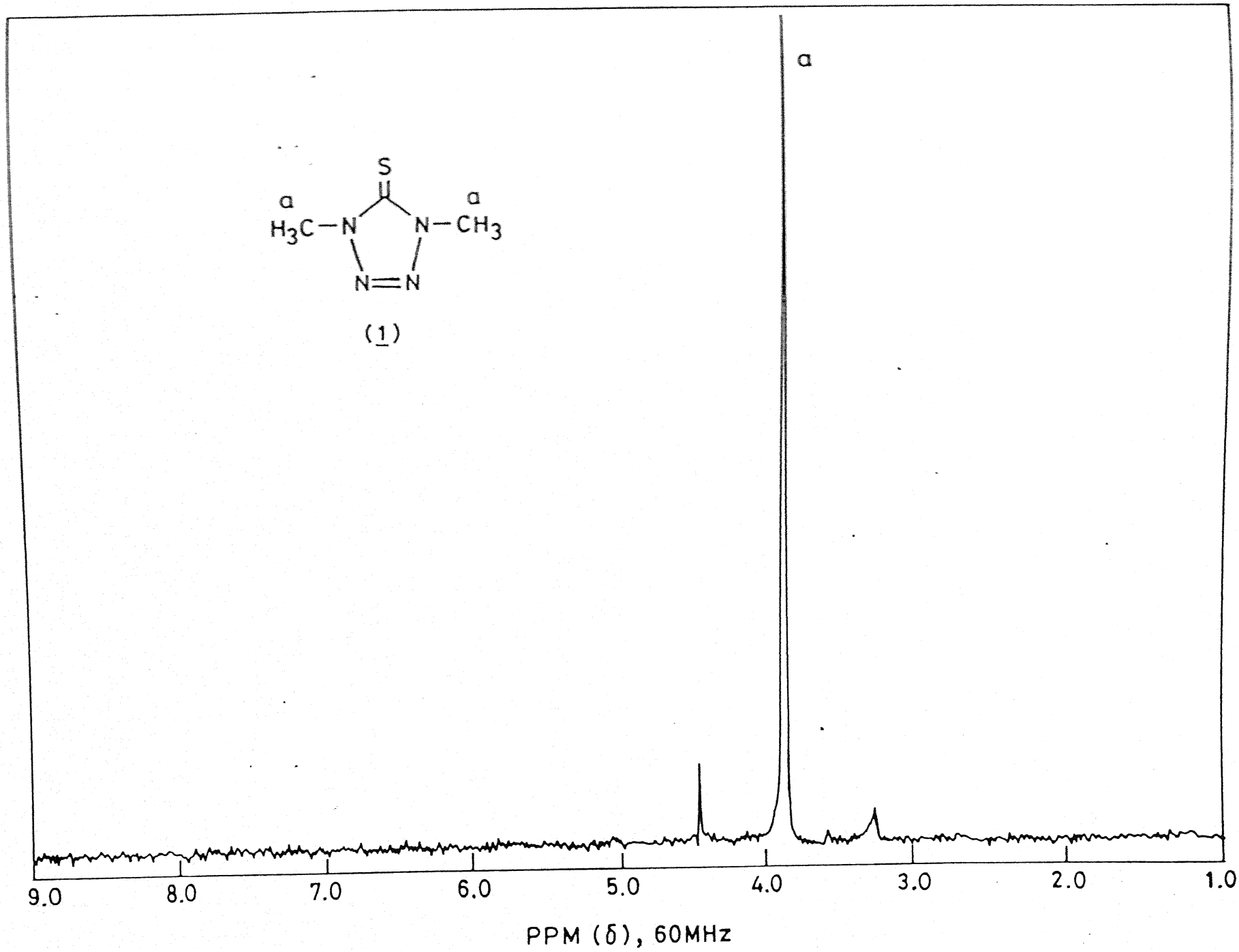
CHART C-25

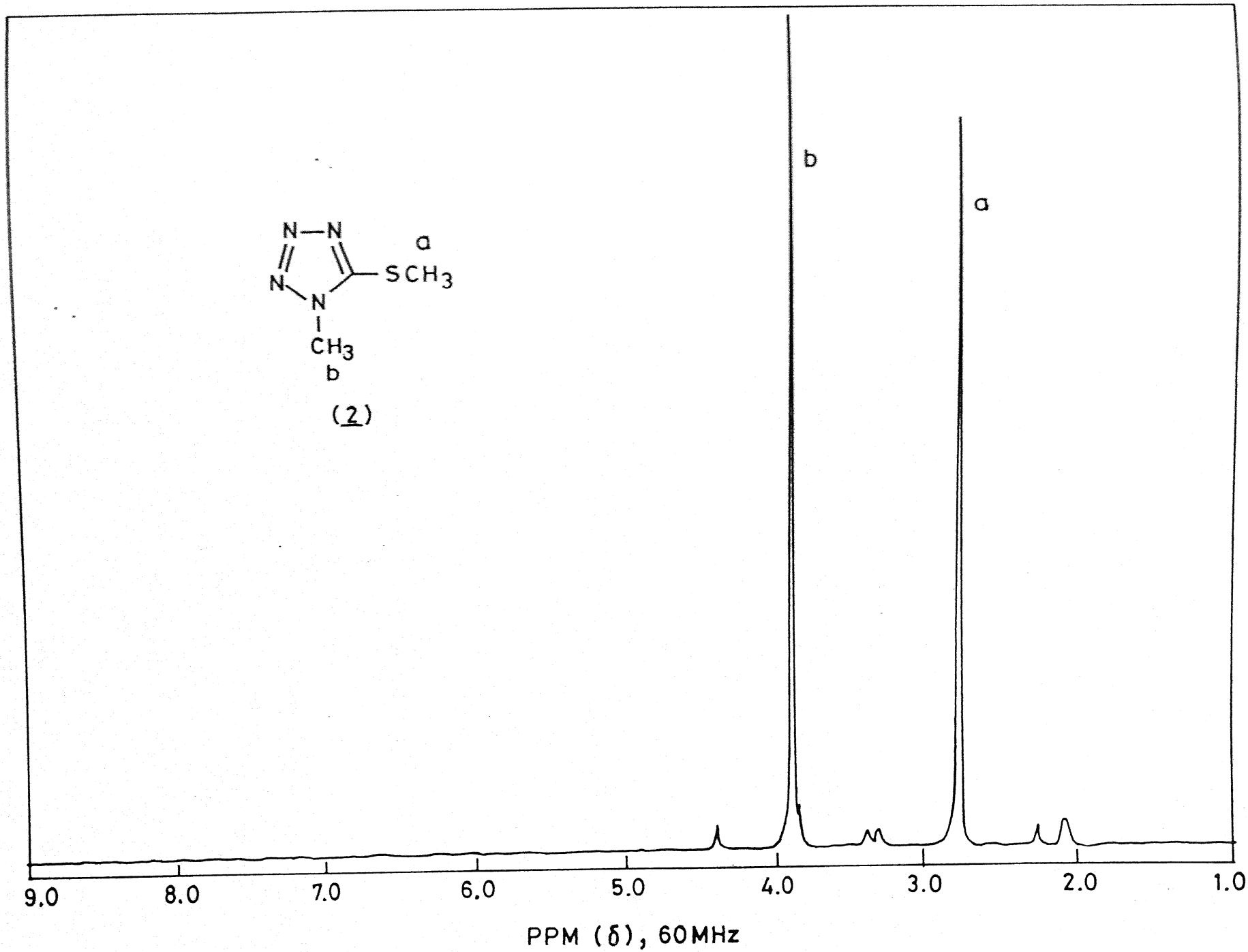


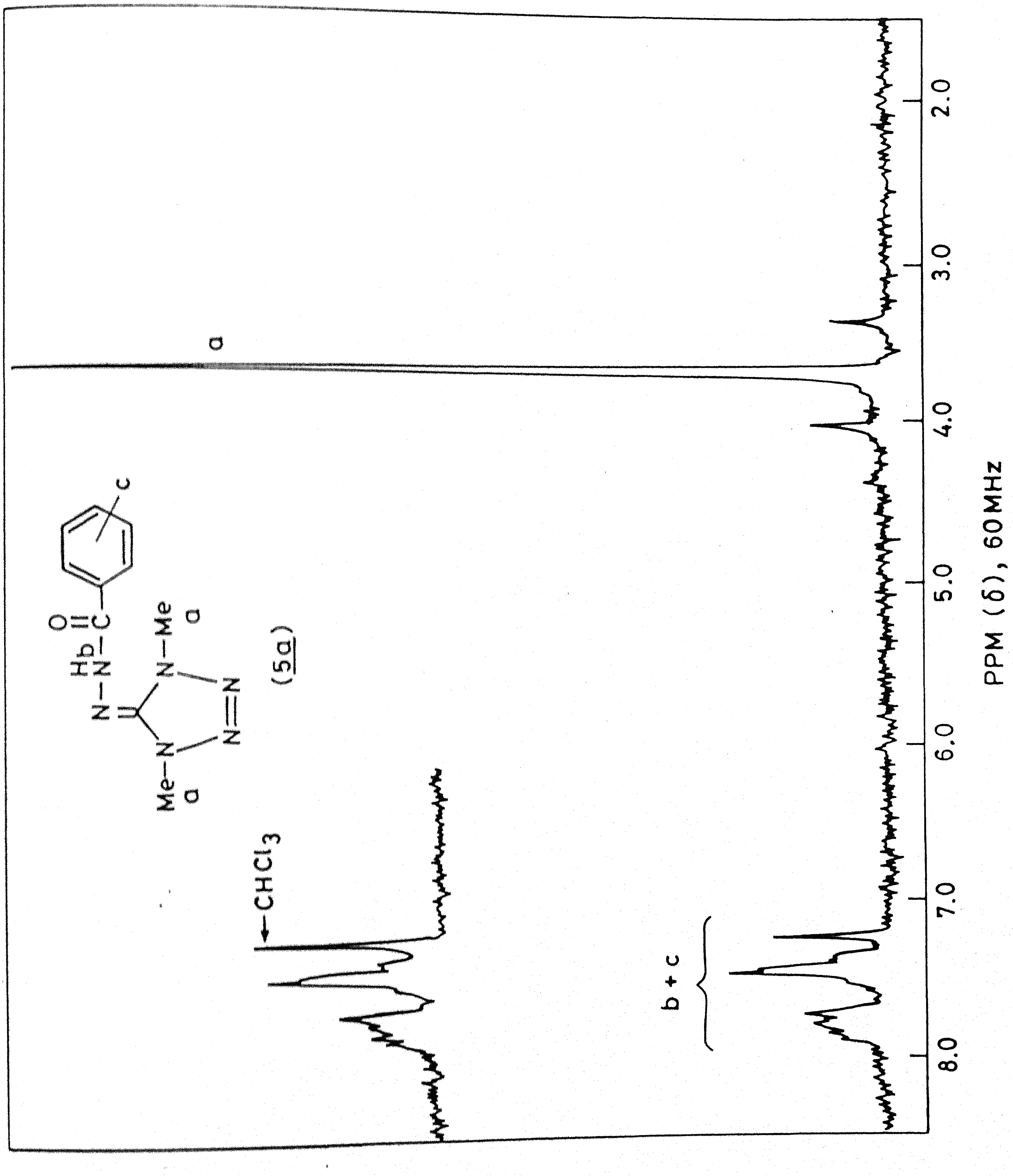
X = Bz Leu OMe X' = Bz - Leu - OH Y = GLY

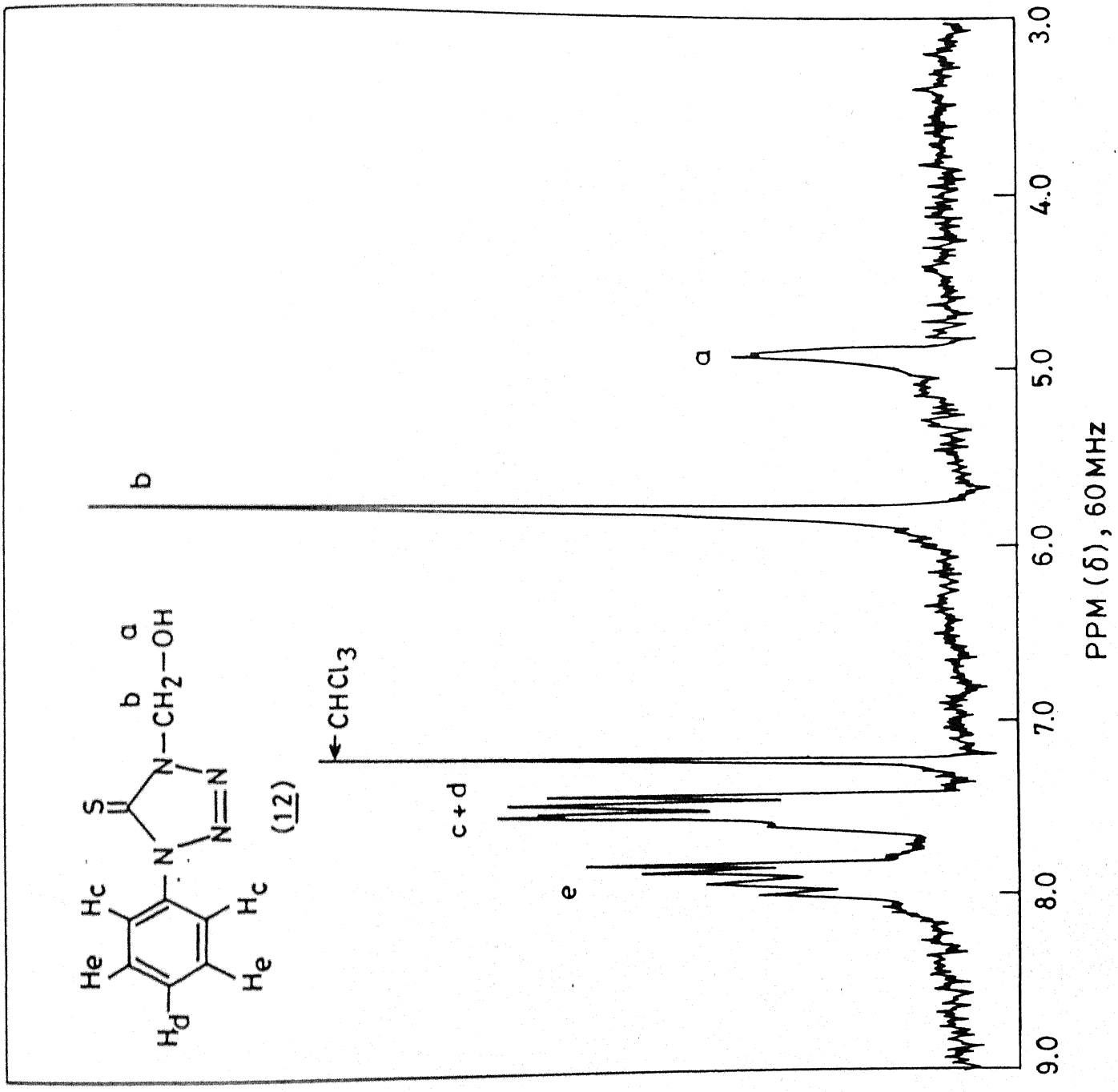
Z = Bz - Leu - GLY - OH

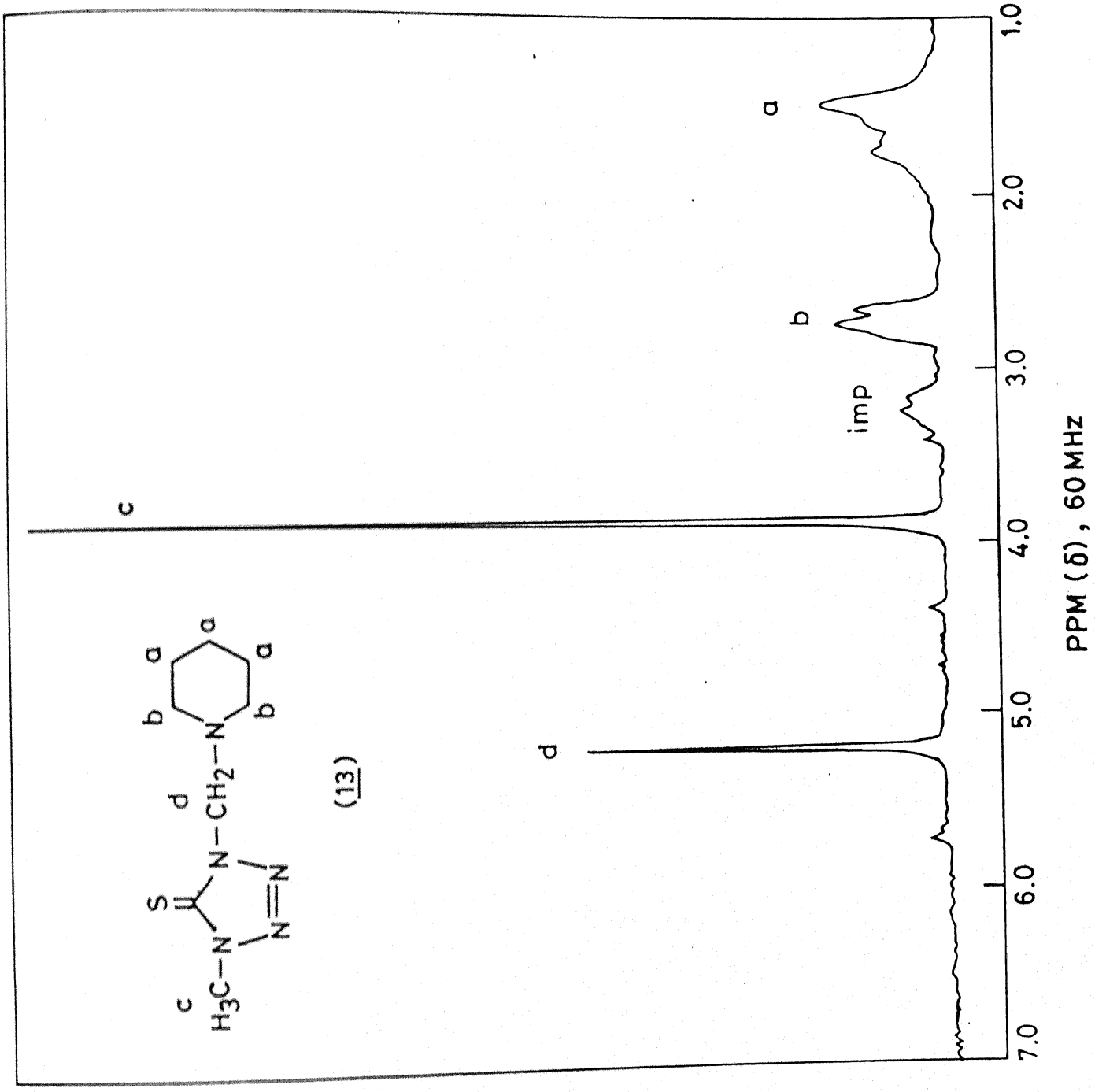
number of experimental frames could be designed to bring about selectivity in peptide bond formation.


a
S
a
a
H_3C-N
$N-CH_3$
$N=N$
(1)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
PPM (δ), 60MHz

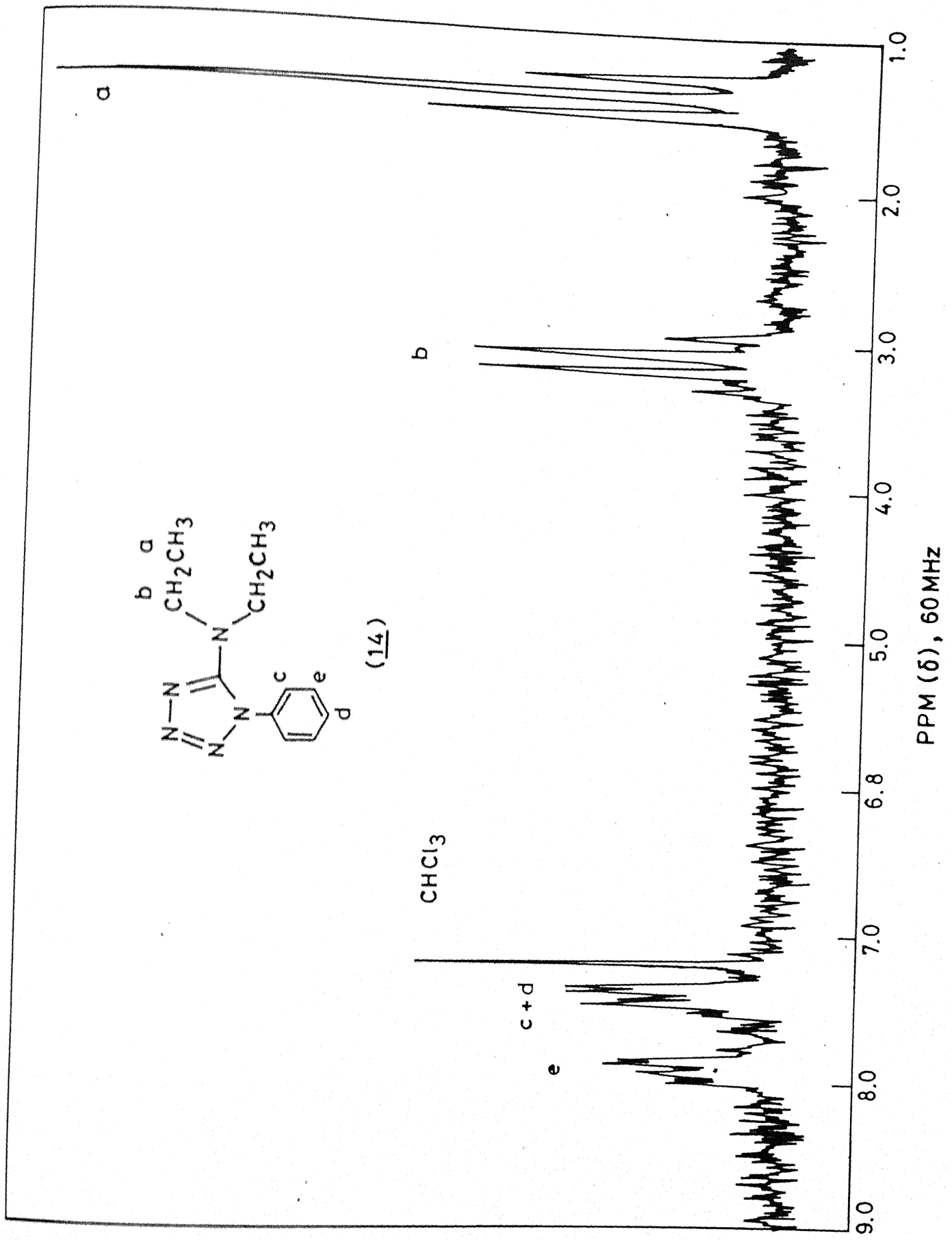

N—N
N
N
SCH3
a
CH3
b
(2)
b
a
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
PPM (δ), 60MHz


a
←CHCl$_3$
b + c
(5a)
PPM (δ), 60MHz
2.0
3.0
4.0
5.0
6.0
7.0
8.0

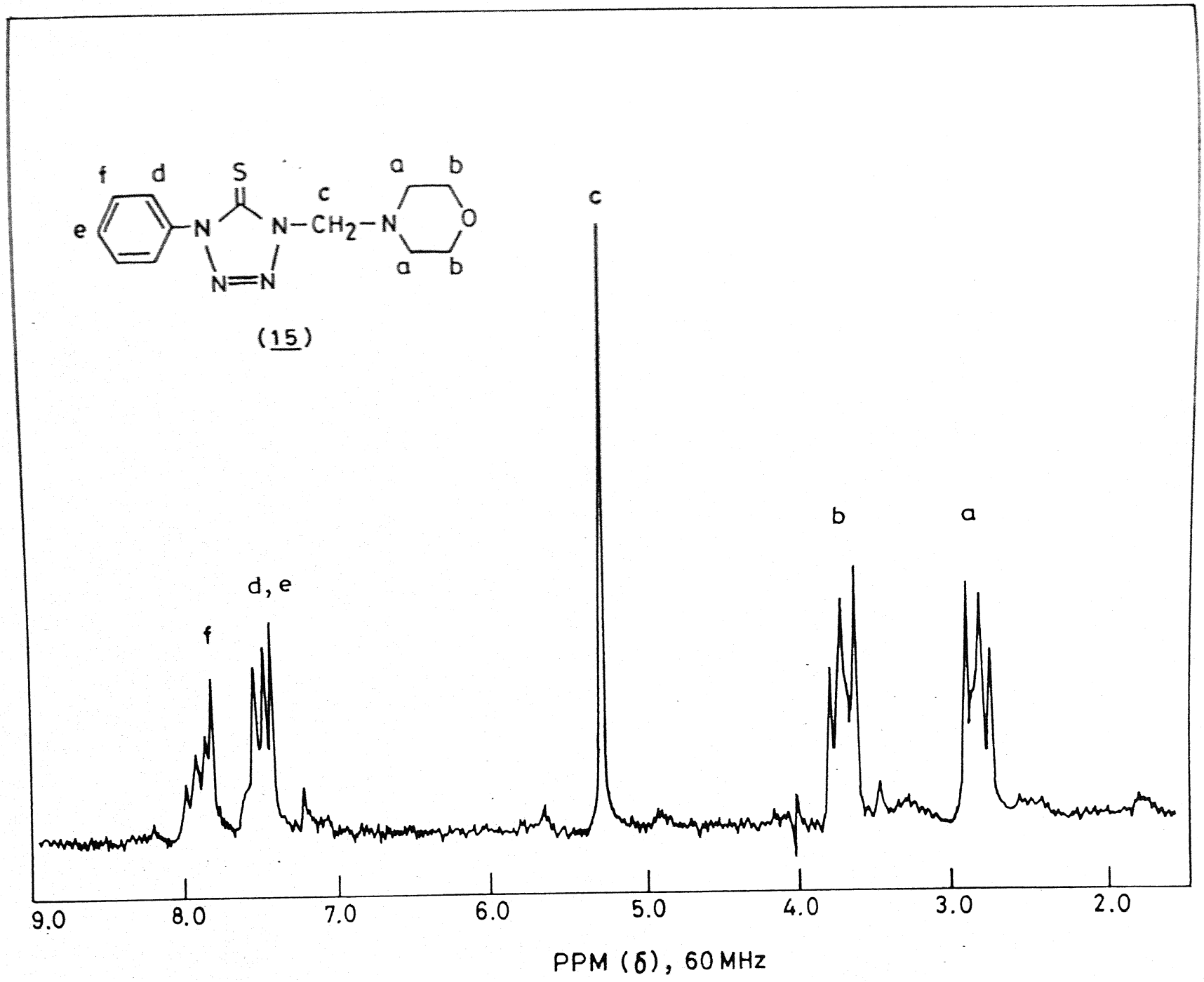

a
b
CH_2-OH
S
N
N
N
N
(12)
H_c
H_c
H_e
H_e
H_d
$CHCl_3$
a
b
c+d
e
3.0
4.0
5.0
6.0
7.0
8.0
9.0
PPM (δ), 60 MHz

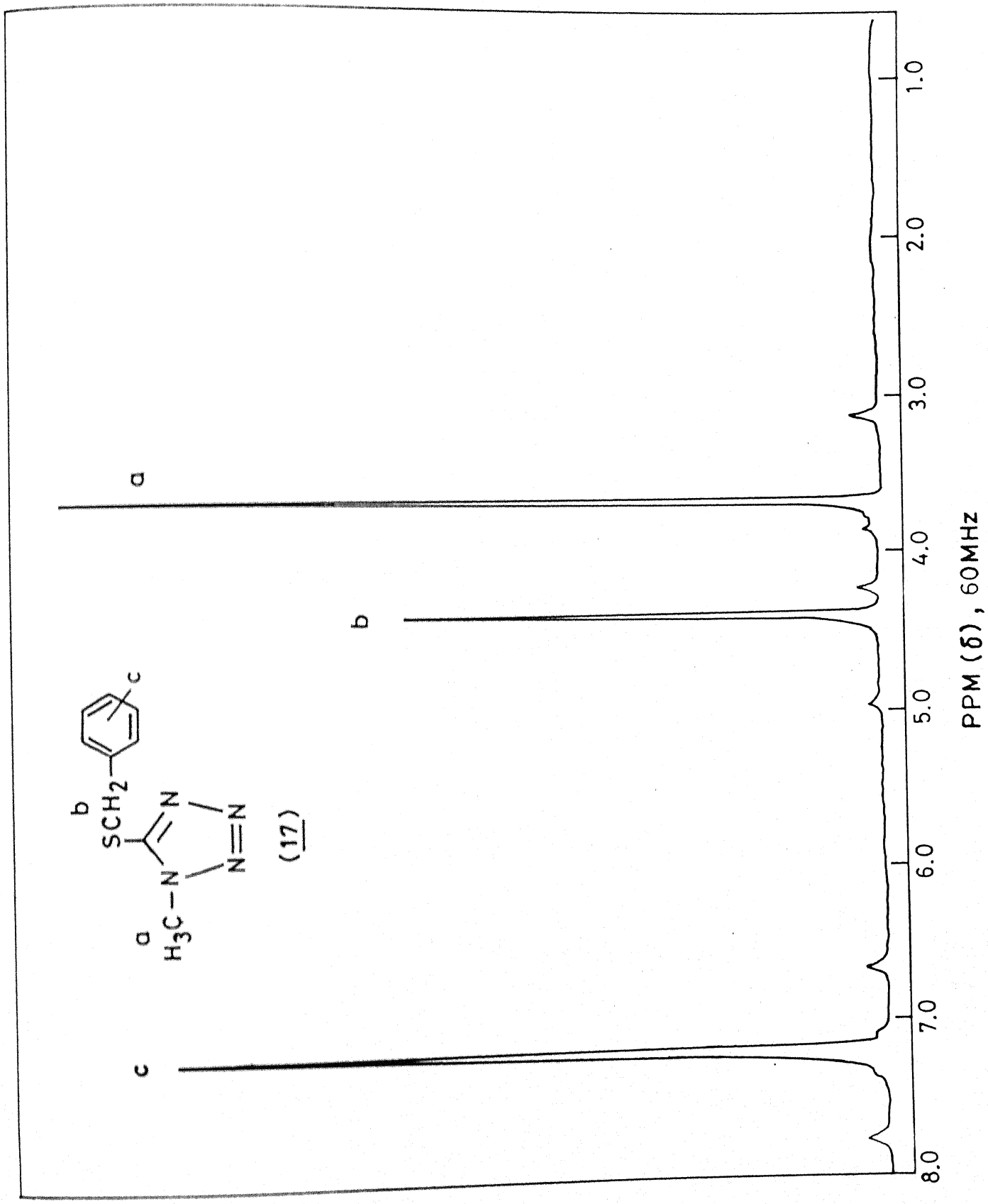


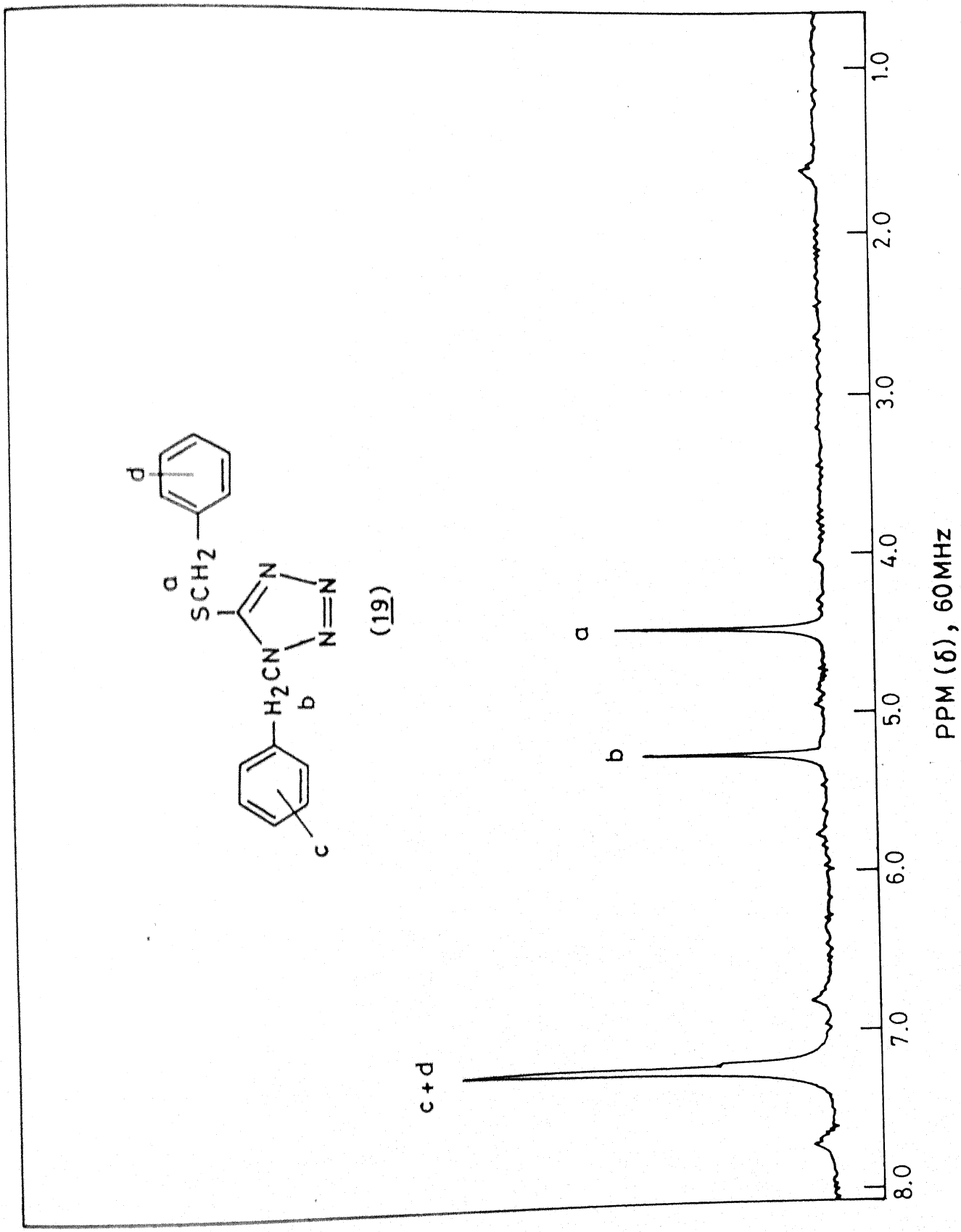
(13)

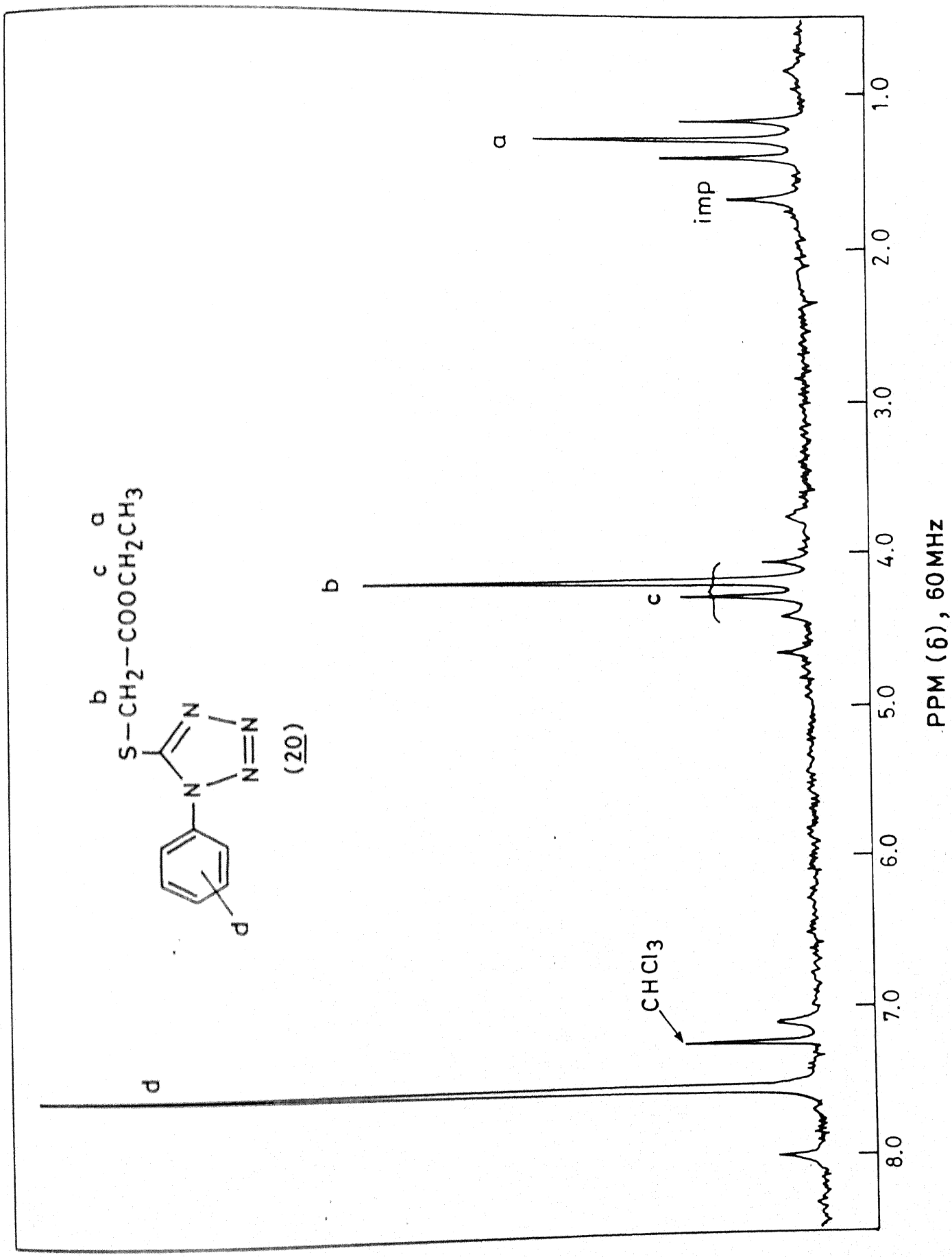

a
b
CHCl3
c+d
e
1.0
2.0
3.0
4.0
5.0
6.8
7.0
8.0
9.0
PPM (δ), 60 MHz
b a
CH2CH3
CH2CH3
N
N
N
N
c
e
d

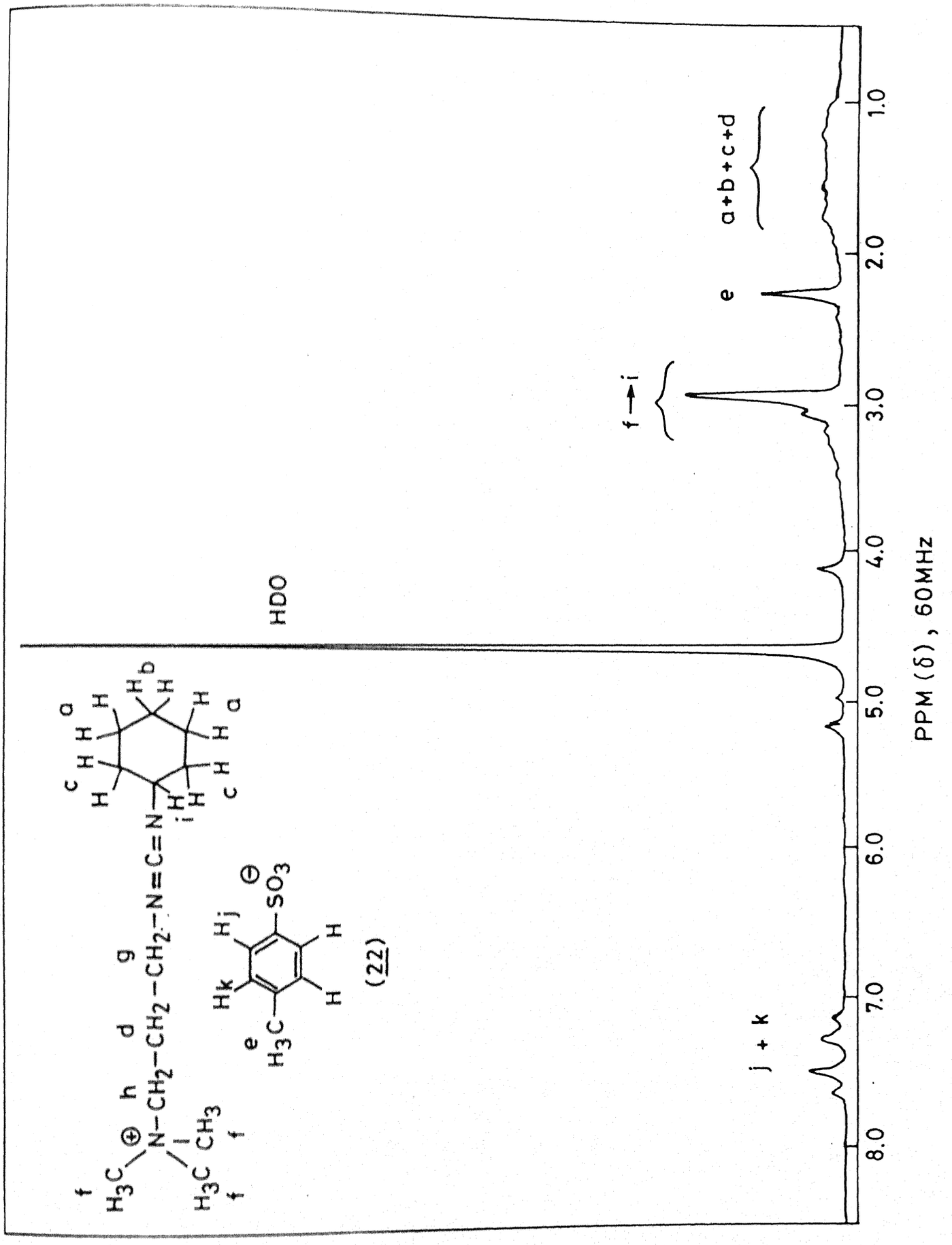
(14)


f
d
S
a
b
c
e
N
N–CH2–N
O
a
b
N=N
(15)
c
b
a
d, e
f
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
PPM (δ), 60 MHz

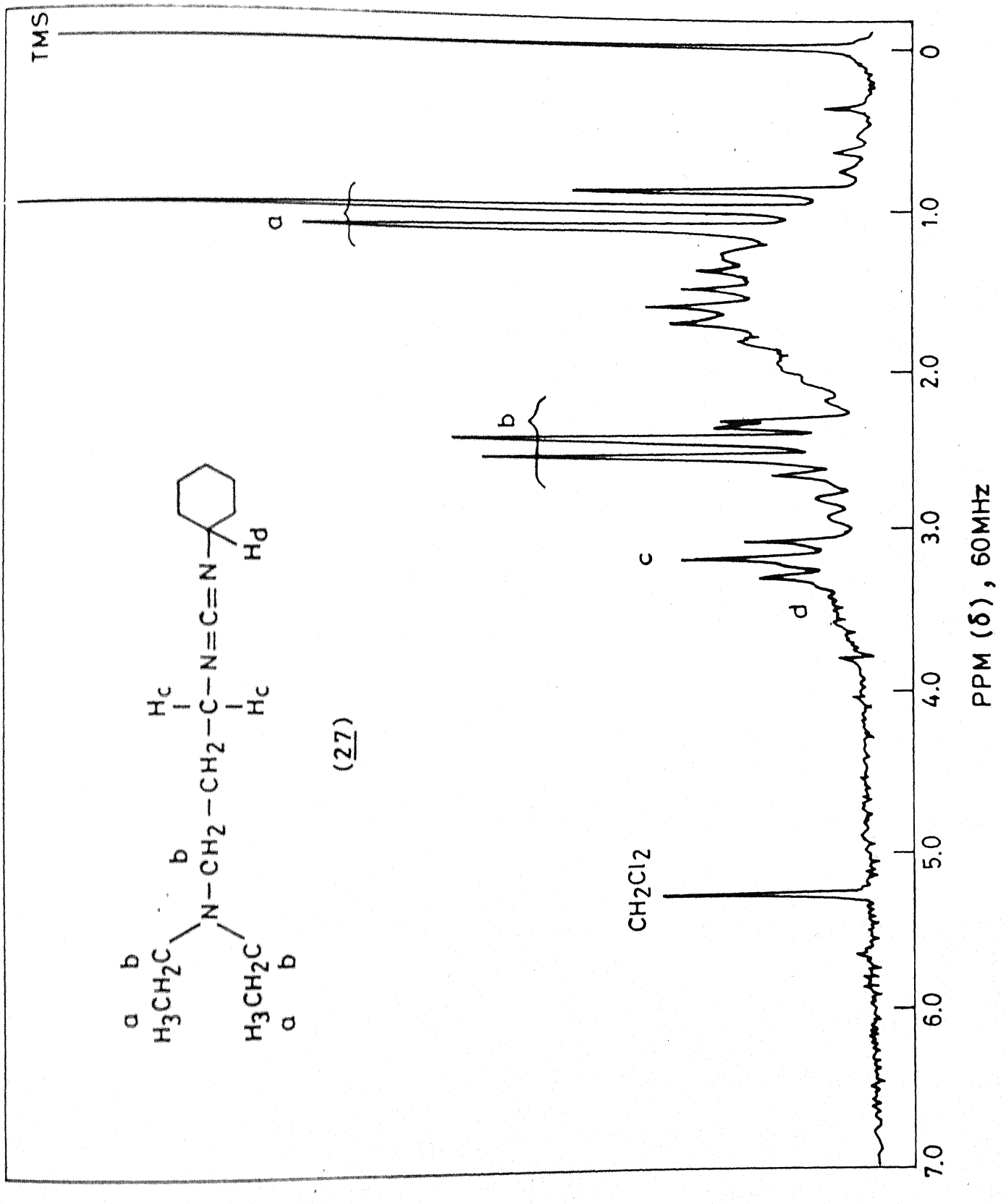

a
b
c
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
PPM (δ), 60MHz
a
H3C–N
b
SCH2
c
(17)


d
a
SCH2
N
N
N
N
H2CN
b
c
(19)
c+d
a
b
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
PPM (δ), 60MHz

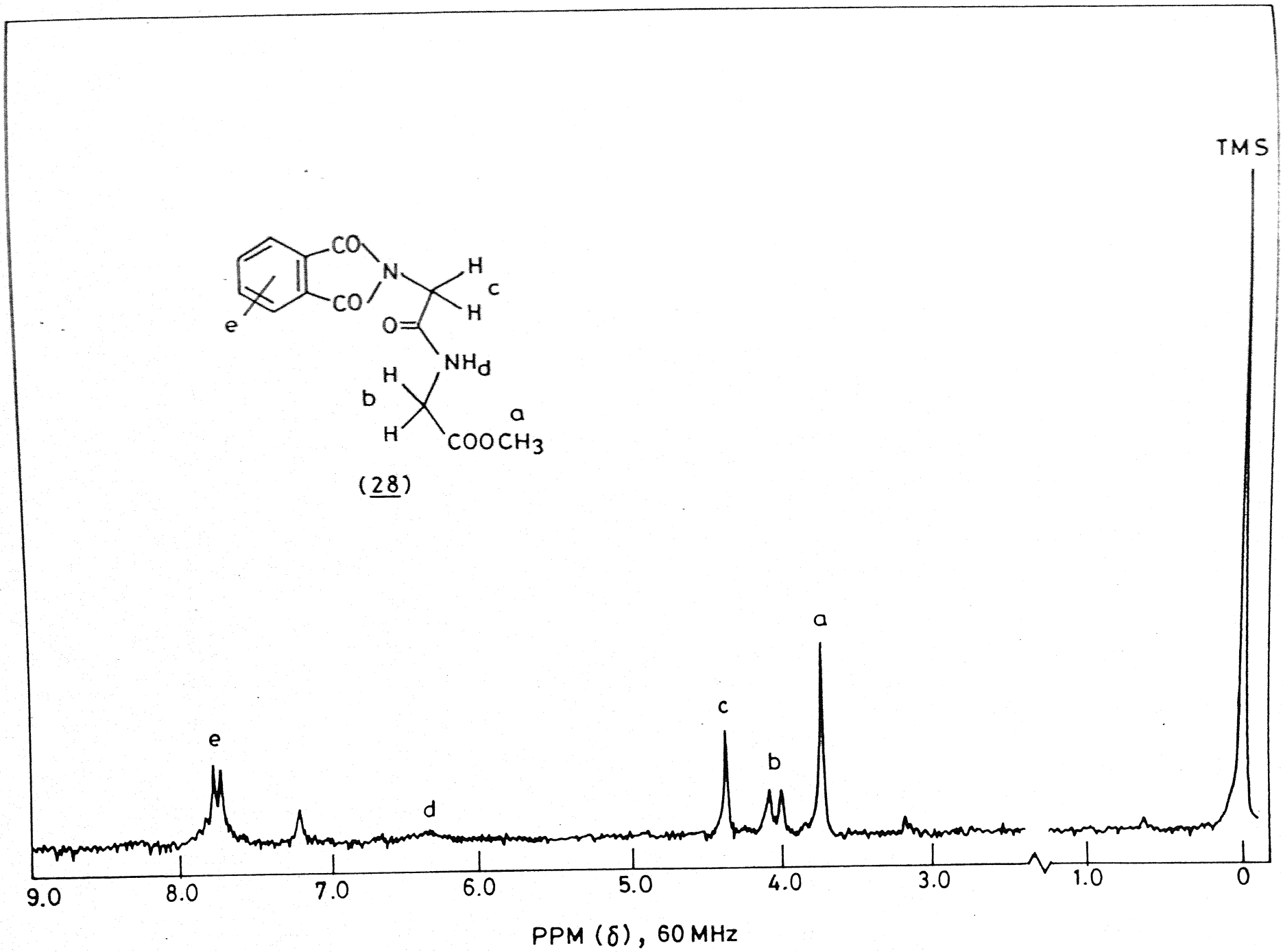

c a
b
S—CH2—COOCH2CH3
N
N
N
N
d
(20)
d
b
a
c
imp
CHCl3
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
PPM (δ), 60 MHz

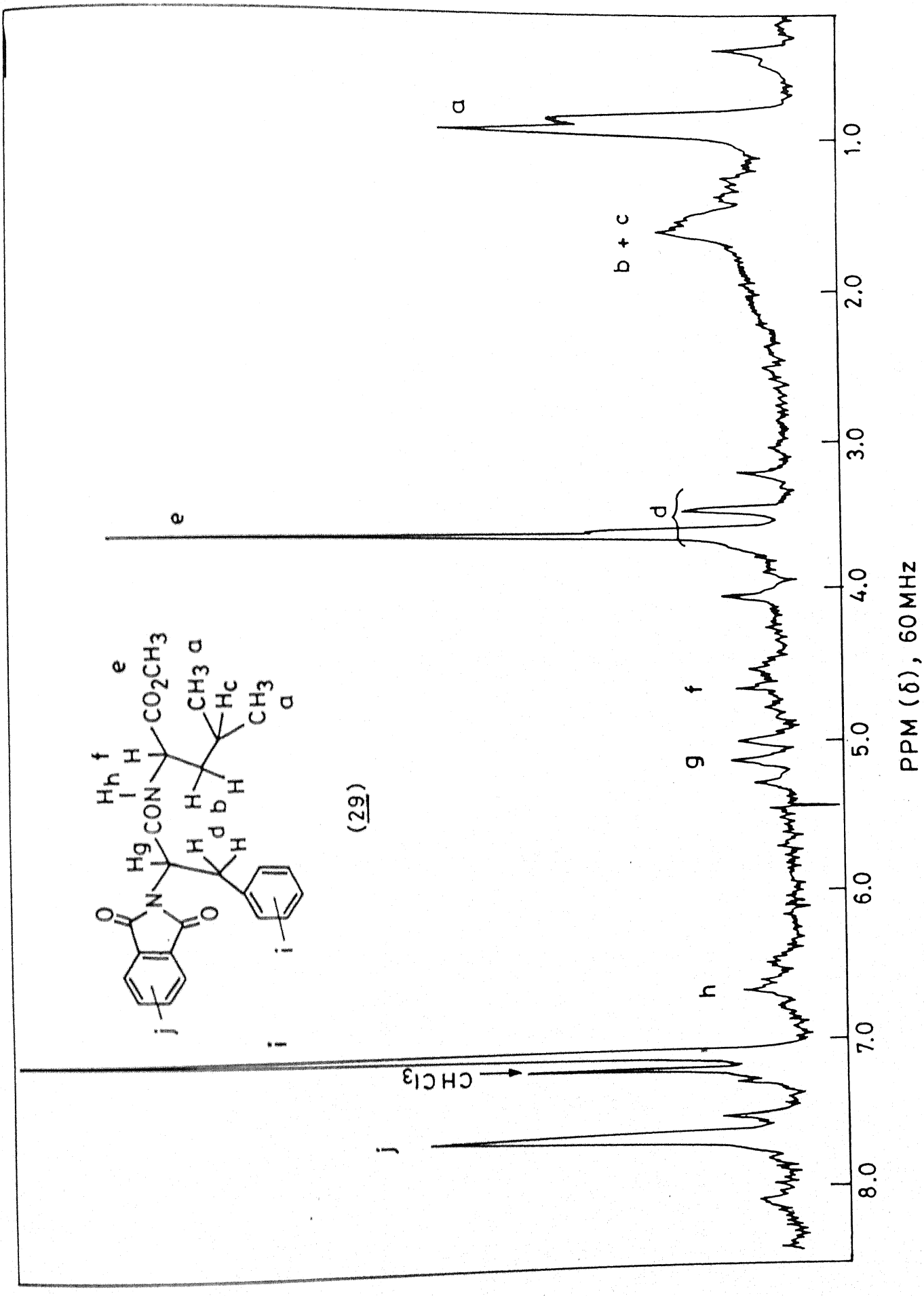


(22)

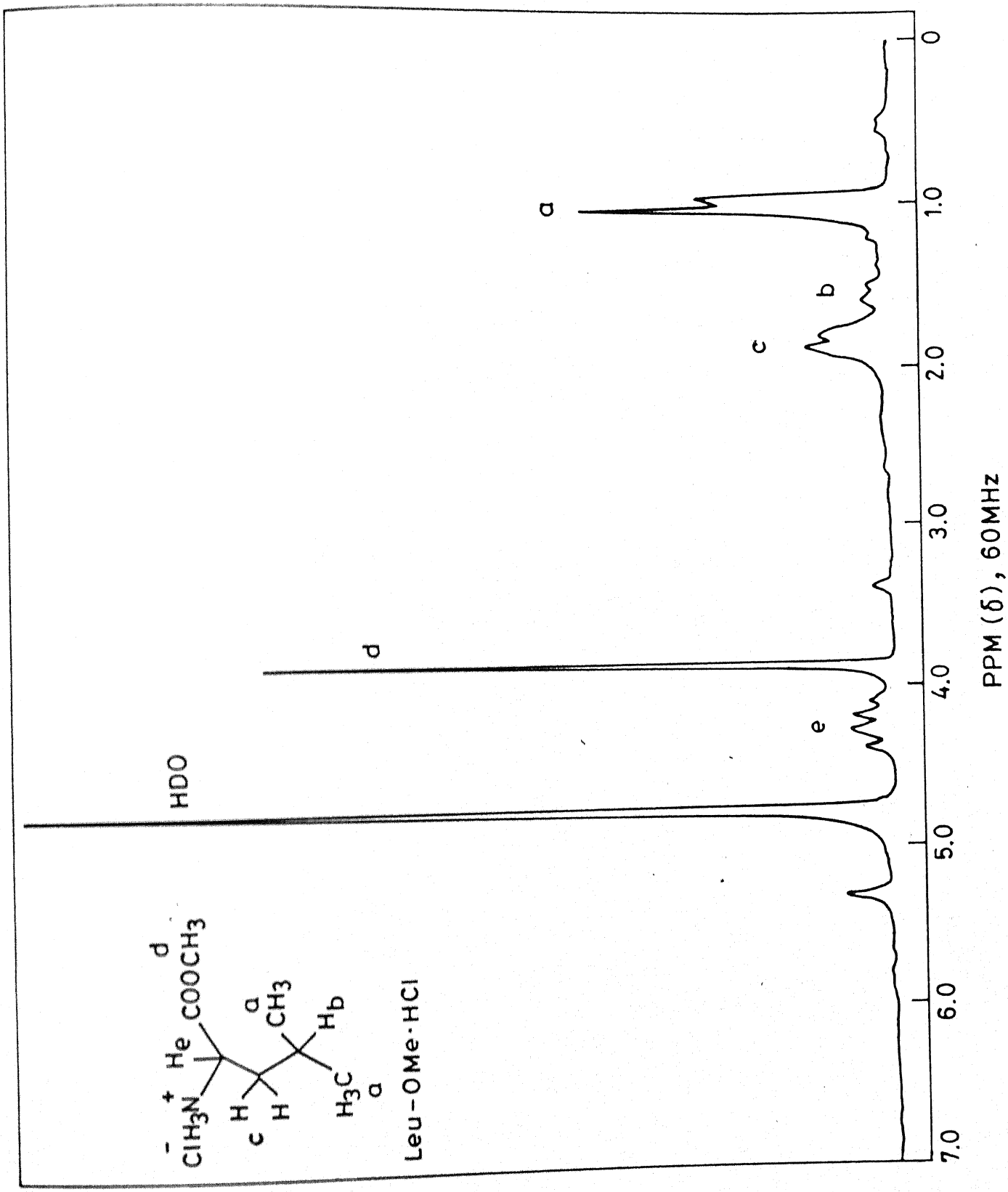


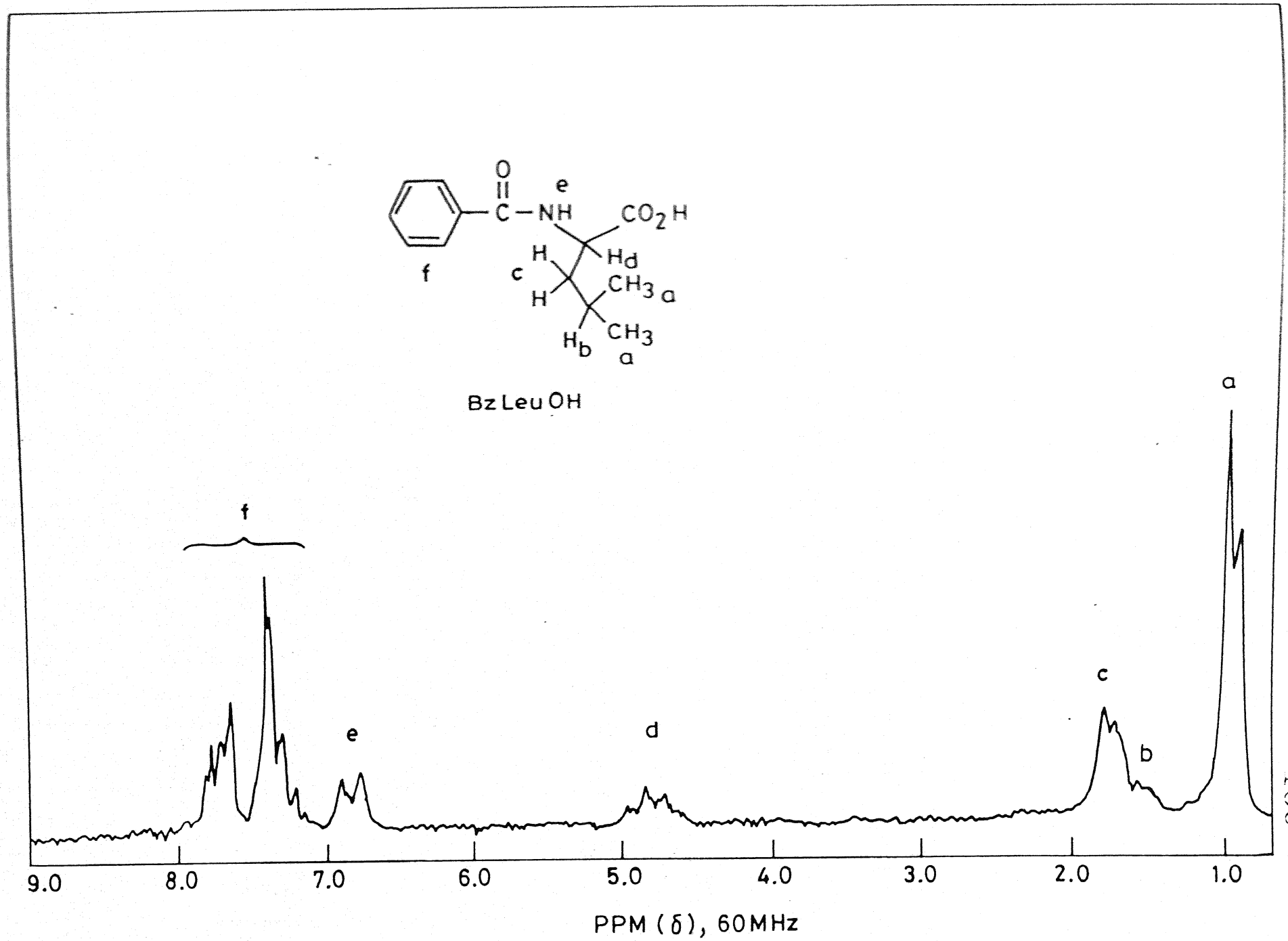
(27)

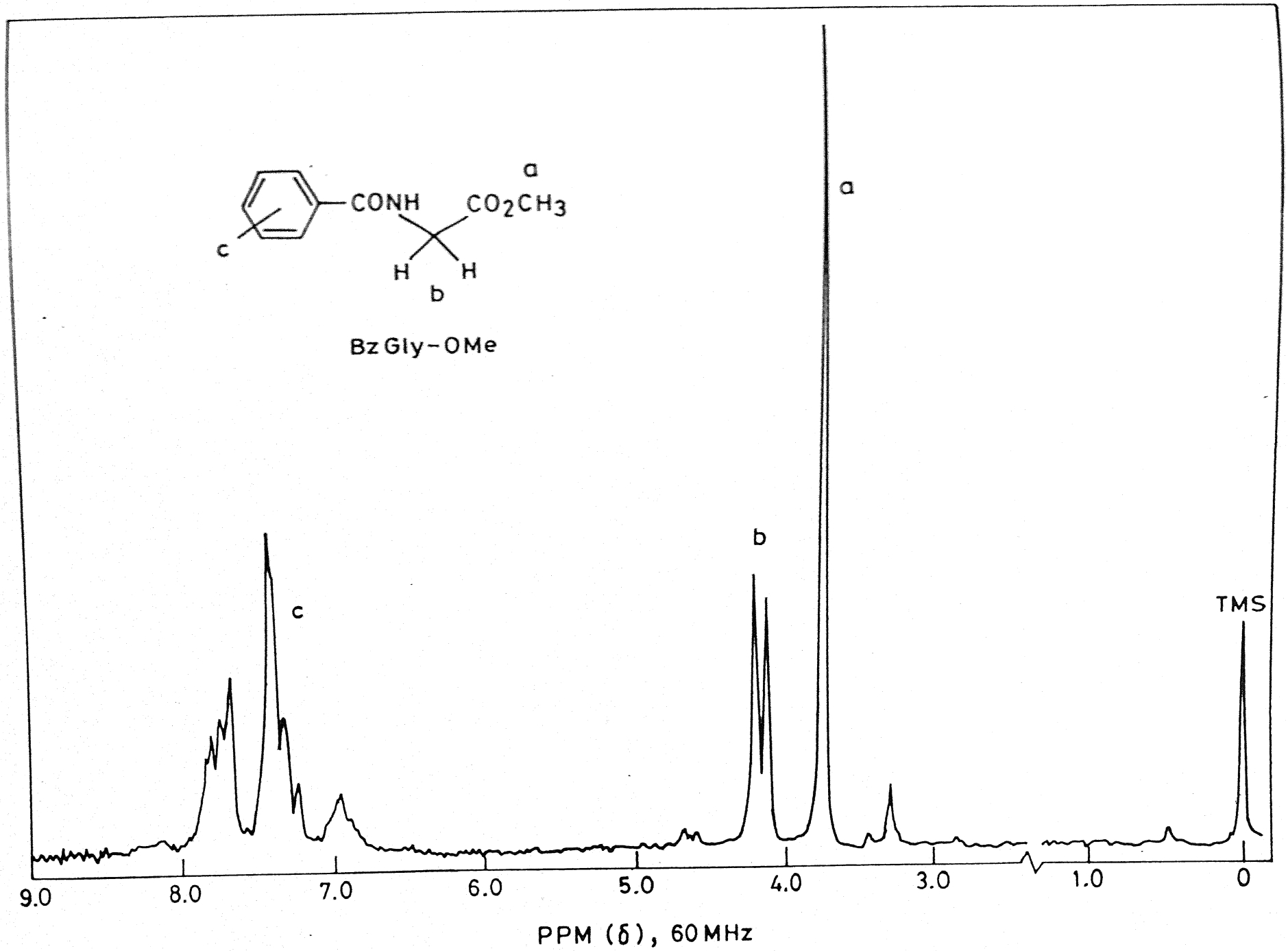

TMS
CO
CO
N
H
c
H
e
O
NHd
H
b
H
a
COOCH3
(28)
a
c
b
e
d
9.0
8.0
7.0
6.0
5.0
4.0
3.0
1.0
0
PPM (δ), 60 MHz

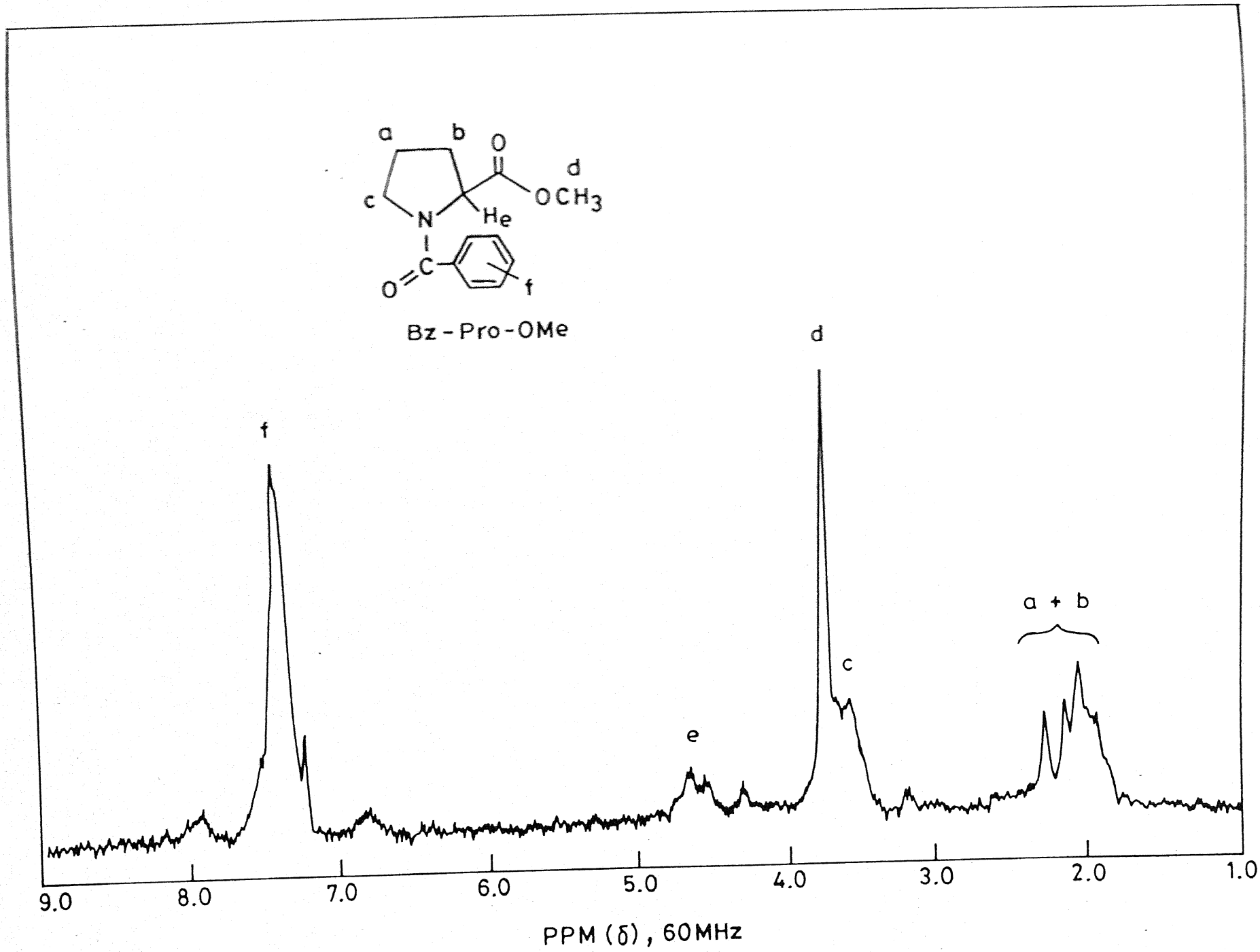


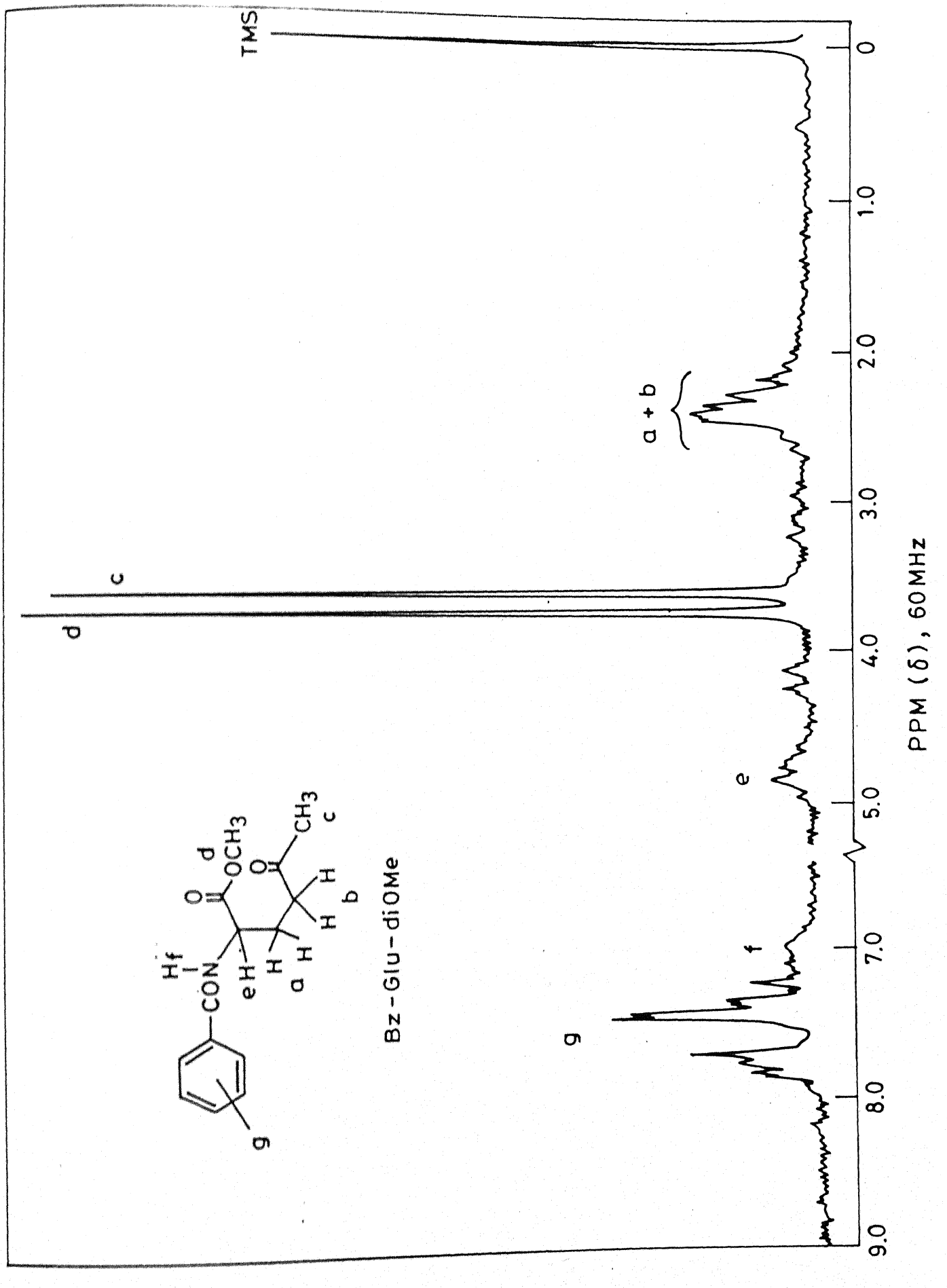
(29)

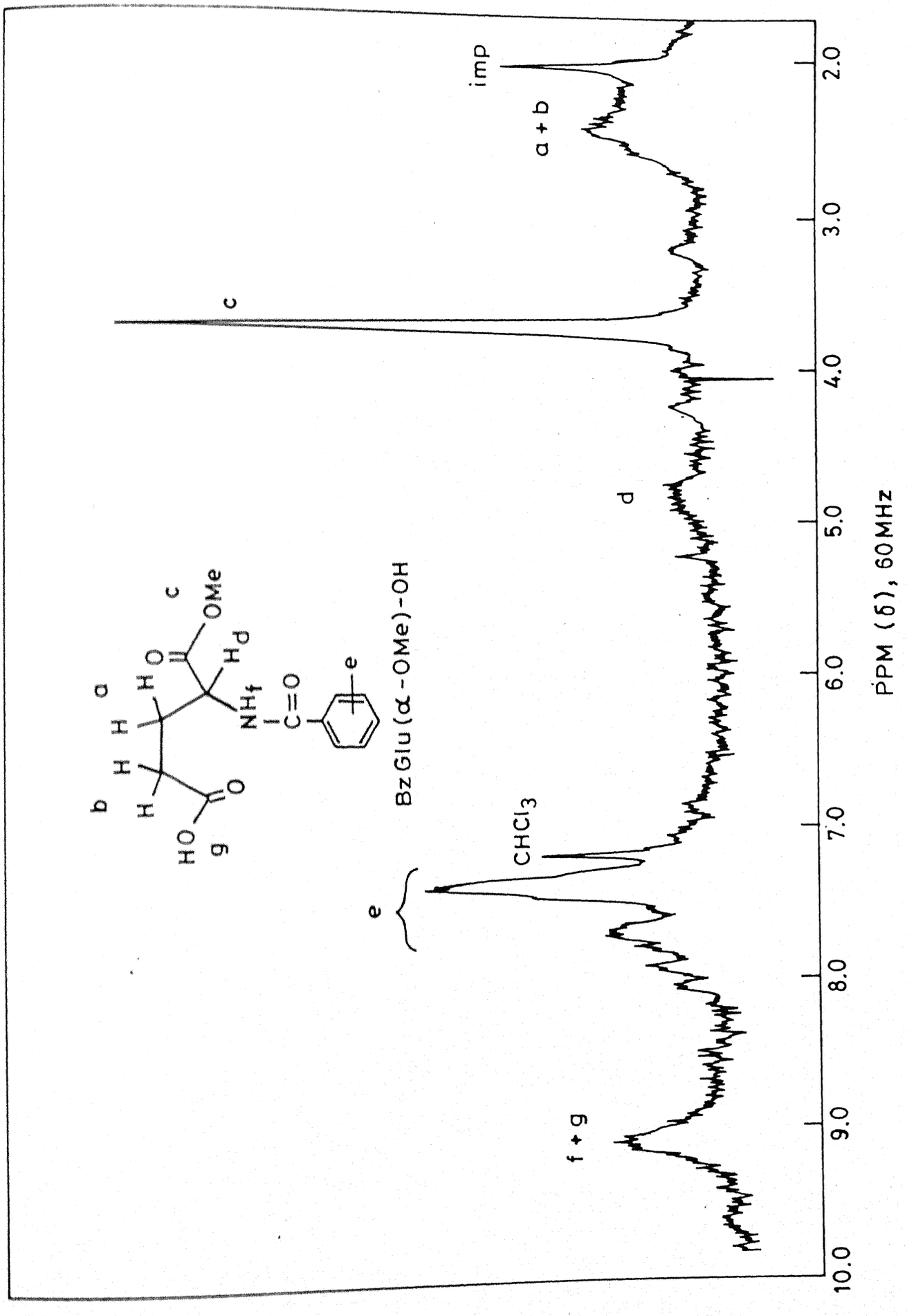

HDO
a
b
c
d
e
PPM (δ), 60MHz
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
Leu-OMe·HCl
$^{-}ClH_3N$
$^{+}$
H_e
$COOCH_3$
d
CH_3
a
H_b
H_3C
a
H
H
c

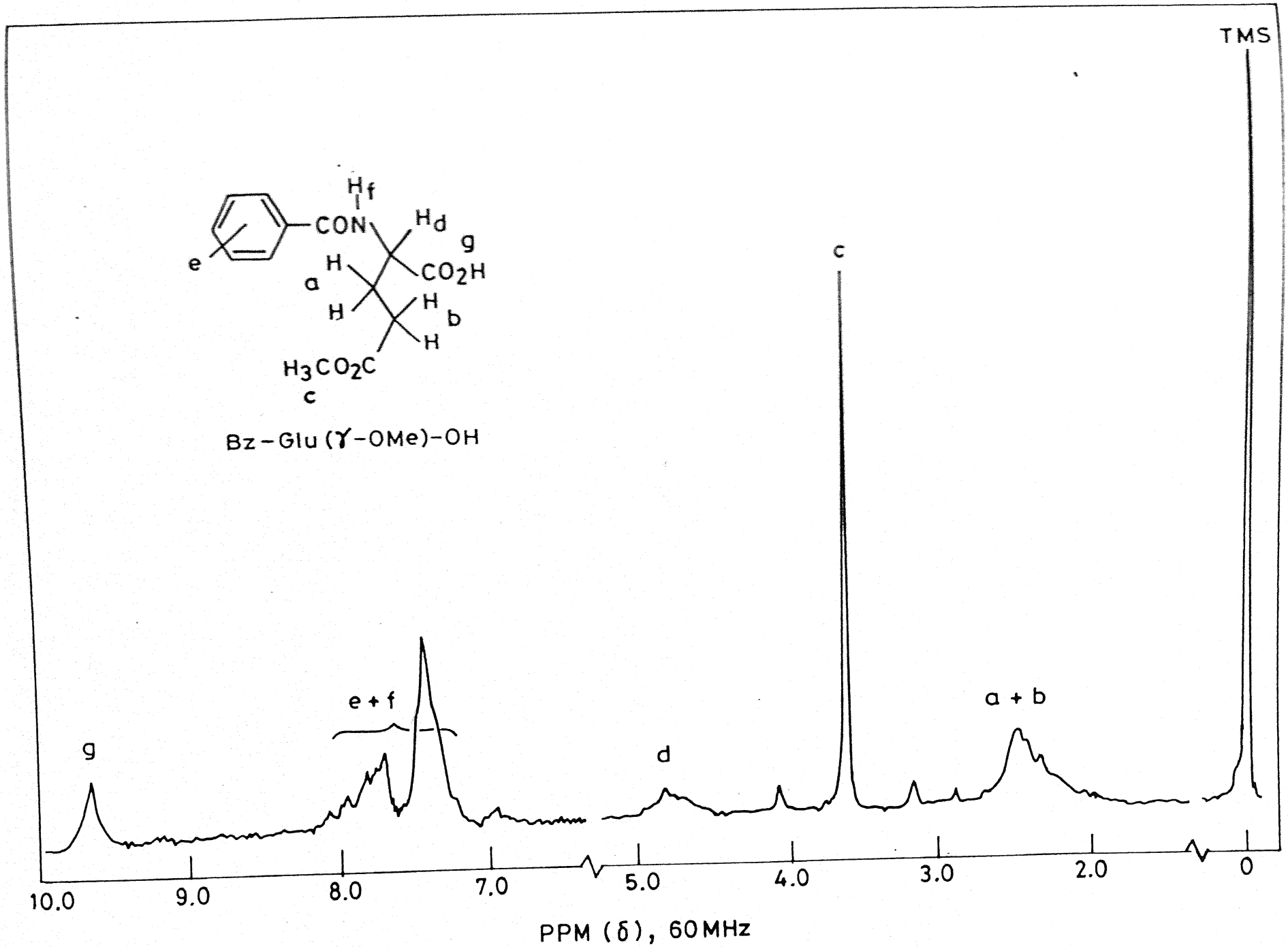

O
e
C – NH
CO2 H
f
c
H
Hd
H
CH3 a
Hb
CH3
a
Bz Leu OH
a
f
c
e
d
b
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
PPM (δ), 60MHz

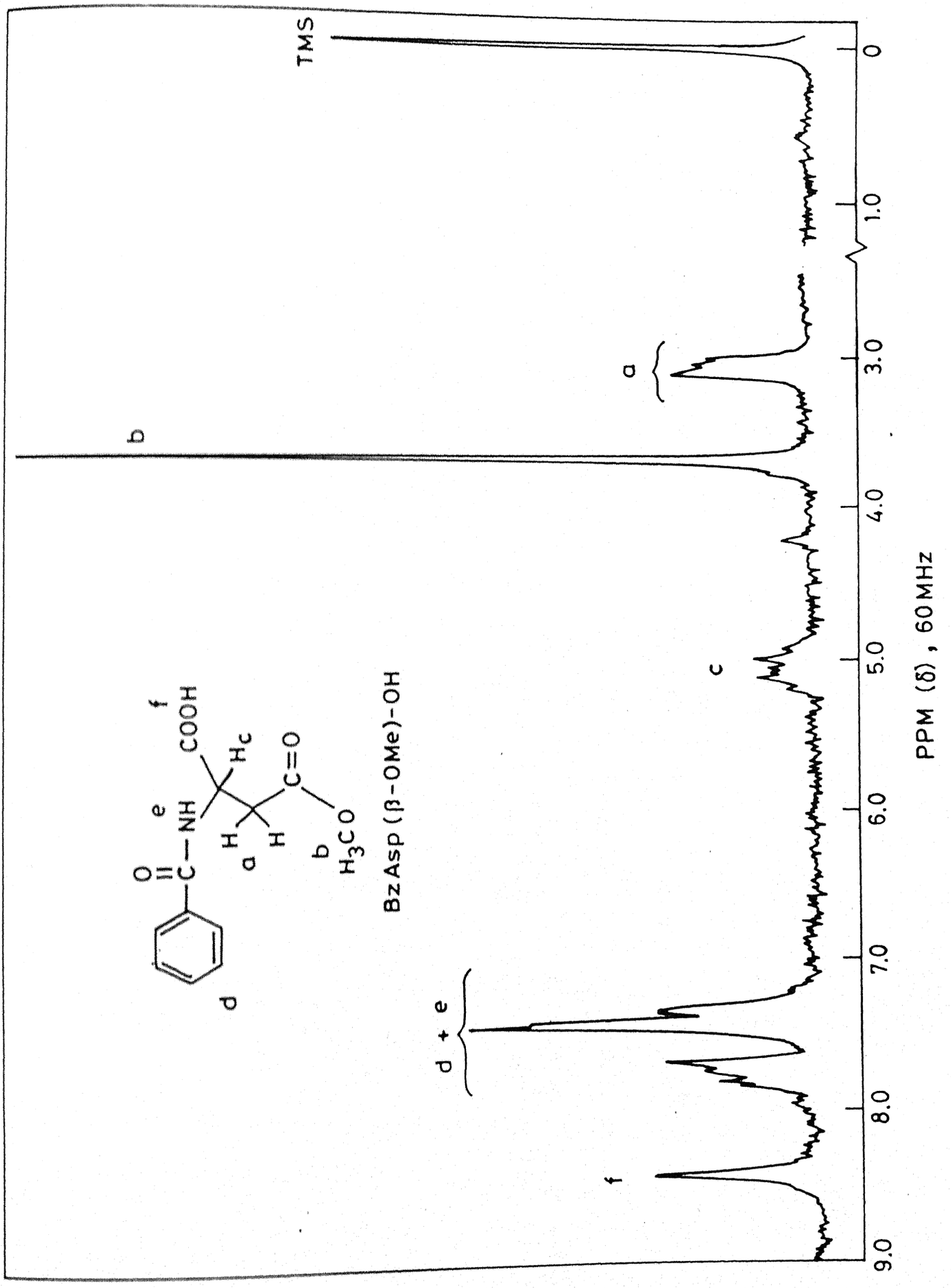

a
CONH
CO2CH3
c
H
H
b
Bz Gly-OMe
a
b
c
TMS
9.0
8.0
7.0
6.0
5.0
4.0
3.0
1.0
0
PPM (δ), 60 MHz

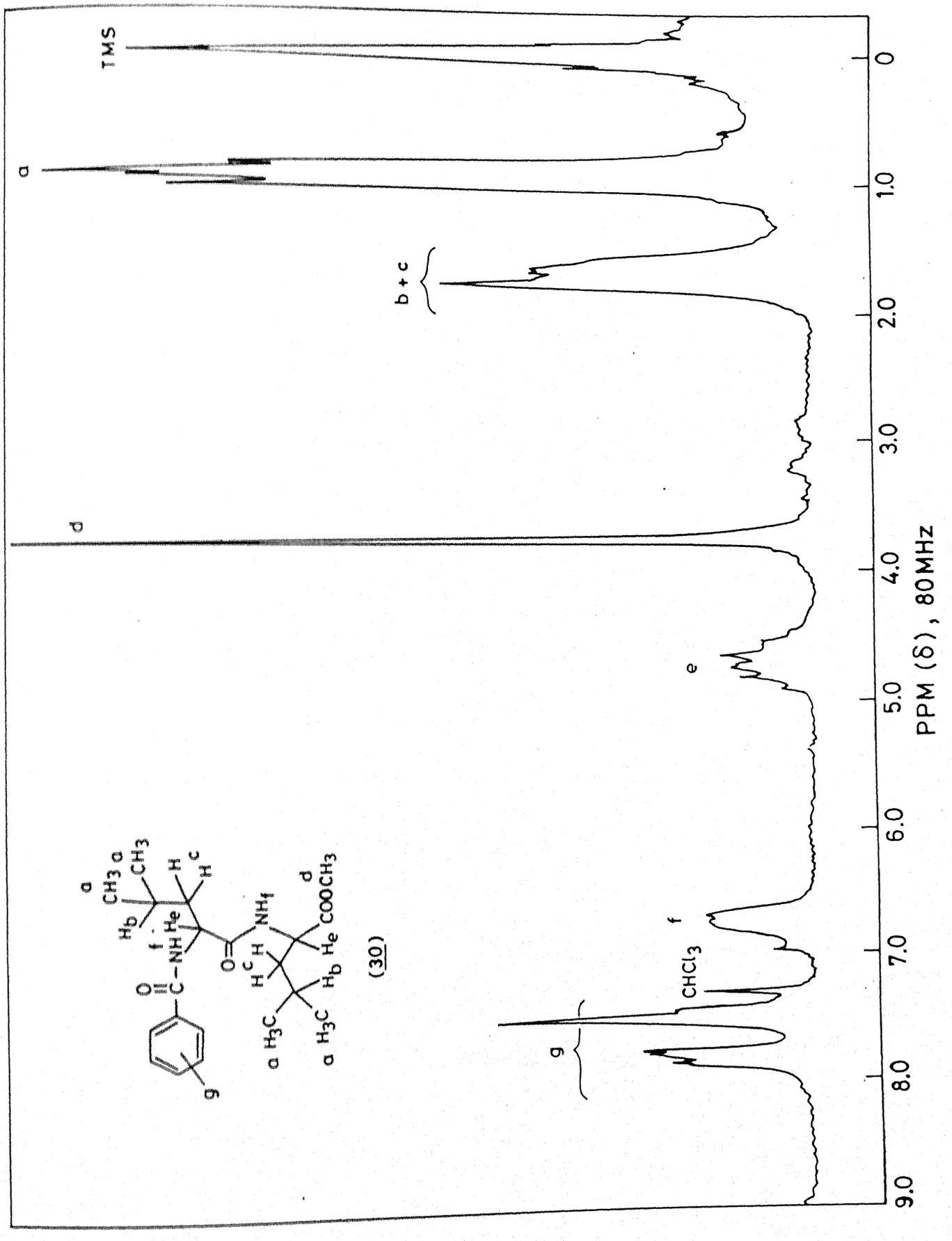

a
b
O
d
c
N
OCH3
He
C
O
f
Bz - Pro - OMe
f
d
e
c
a + b
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
PPM (δ), 60MHz


TMS
d
c
a + b
e
f
g
9.0
8.0
7.0
5.0
4.0
3.0
2.0
1.0
0
PPM (δ), 60MHz
Bz-Glu-diOMe


Bz Glu (α-OMe)-OH
PPM (δ), 60 MHz
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
f + g
e
CHCl3
d
c
a + b
imp


TMS
Hf
CON
Hd
g
e
H
a
CO2H
H
H
b
H
H3CO2C
c
Bz-Glu (γ-OMe)-OH
c
e + f
a + b
g
d
10.0
9.0
8.0
7.0
5.0
4.0
3.0
2.0
0
PPM (δ), 60MHz


TMS
b
a
c
d + e
f
9.0
8.0
7.0
6.0
5.0
4.0
3.0
1.0
0
PPM (δ), 60 MHz
BzAsp (β-OMe)-OH
O
C
NH
e
f
COOH
Hc
H
a
H
C=O
b
H3CO
d



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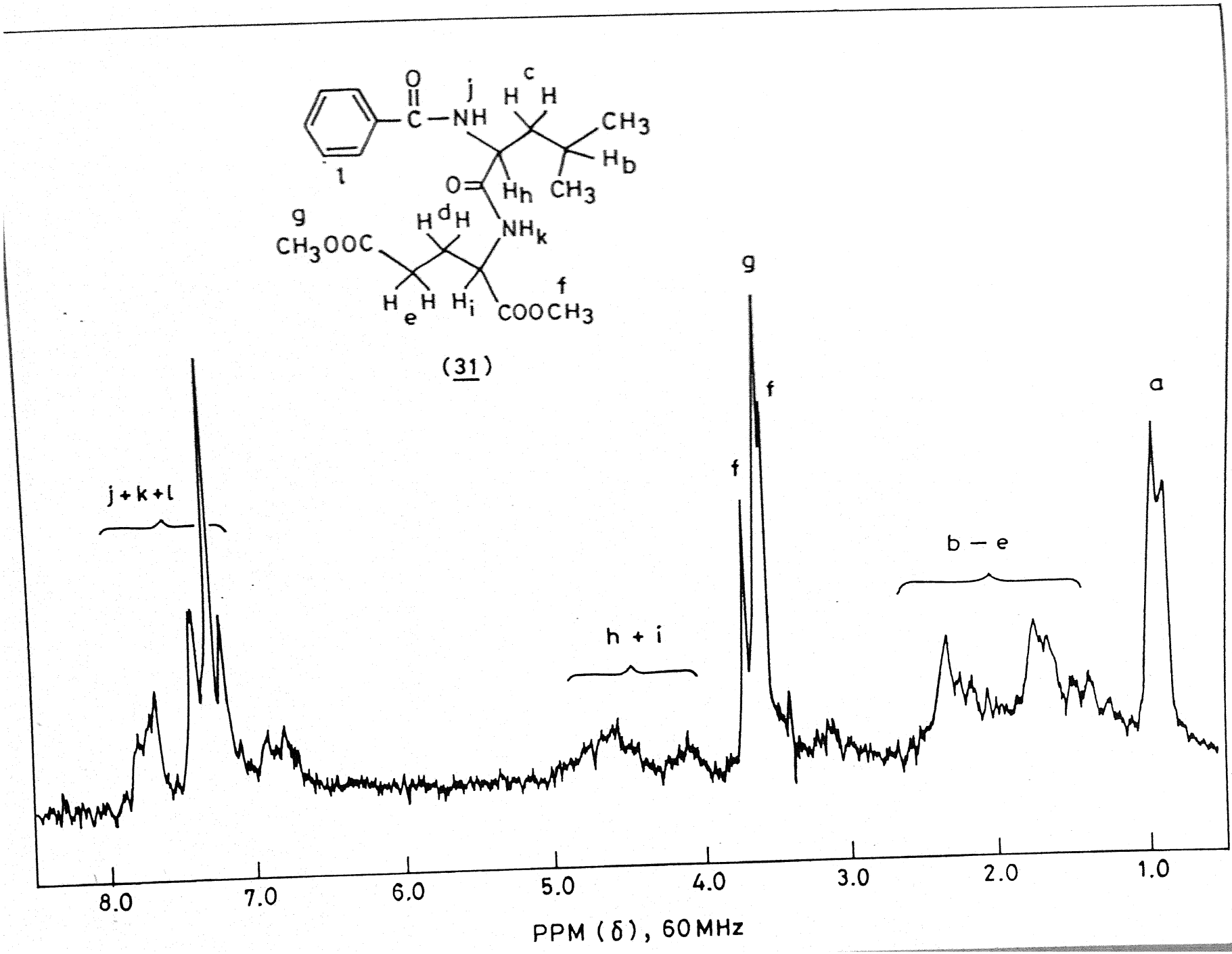
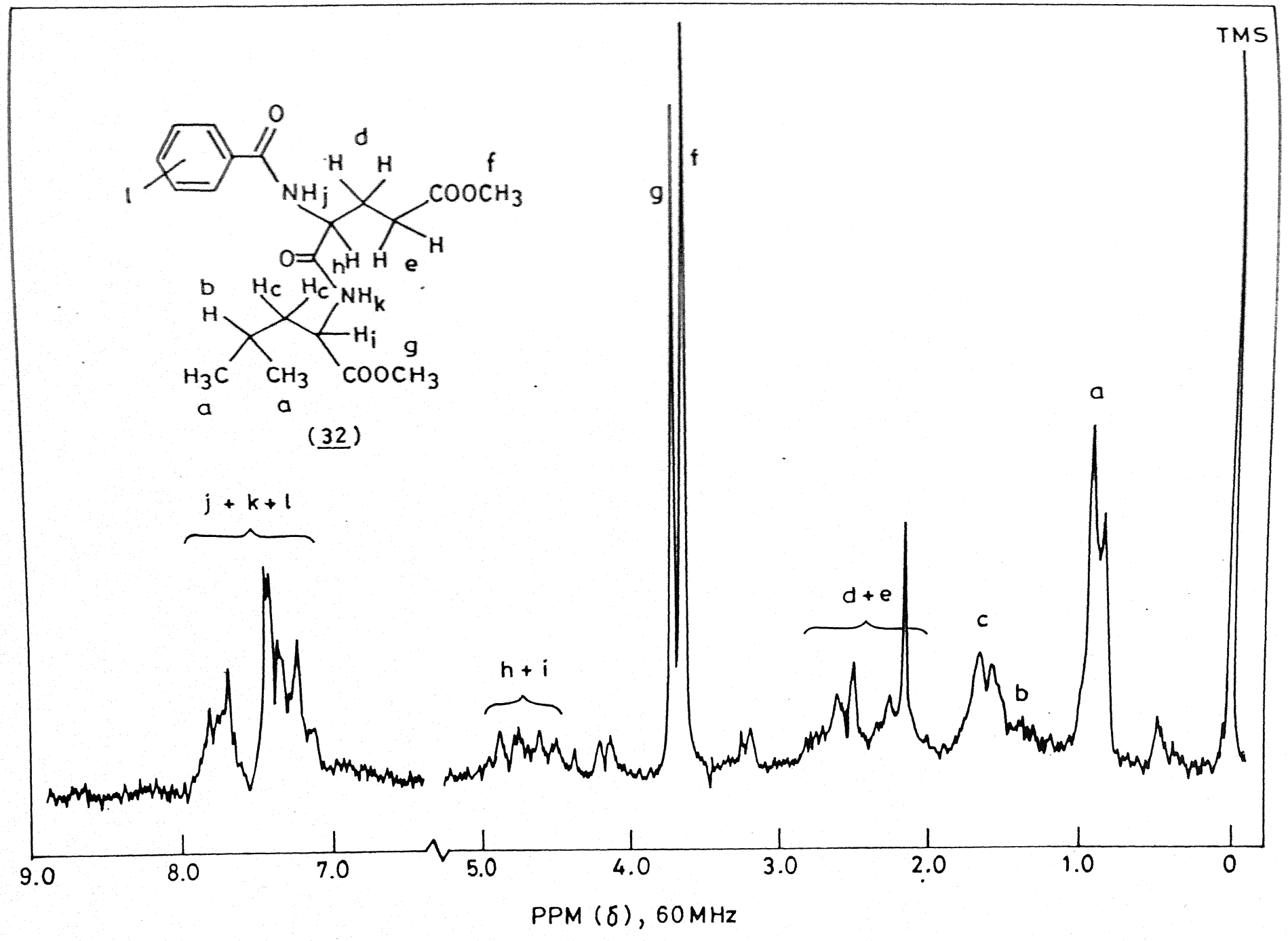

O
j
c
C—NH
H H
CH3
Hb
1
O=
Hh
CH3
g
CH3OOC
H d H
NHk
He H Hi
f
COOCH3
(31)
g
f
f
a
j+k+l
h+i
b—e
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
PPM (δ), 60 MHz



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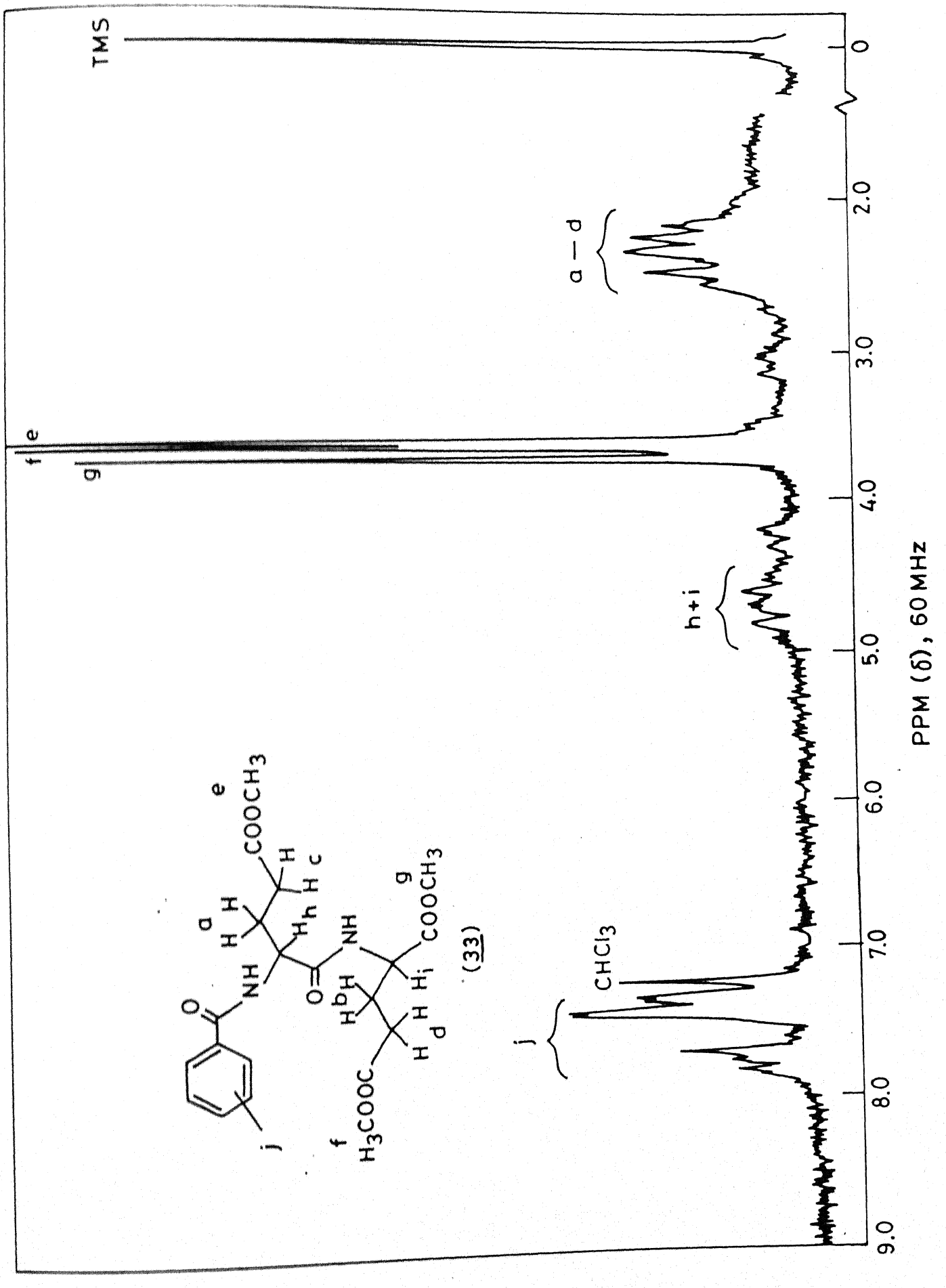
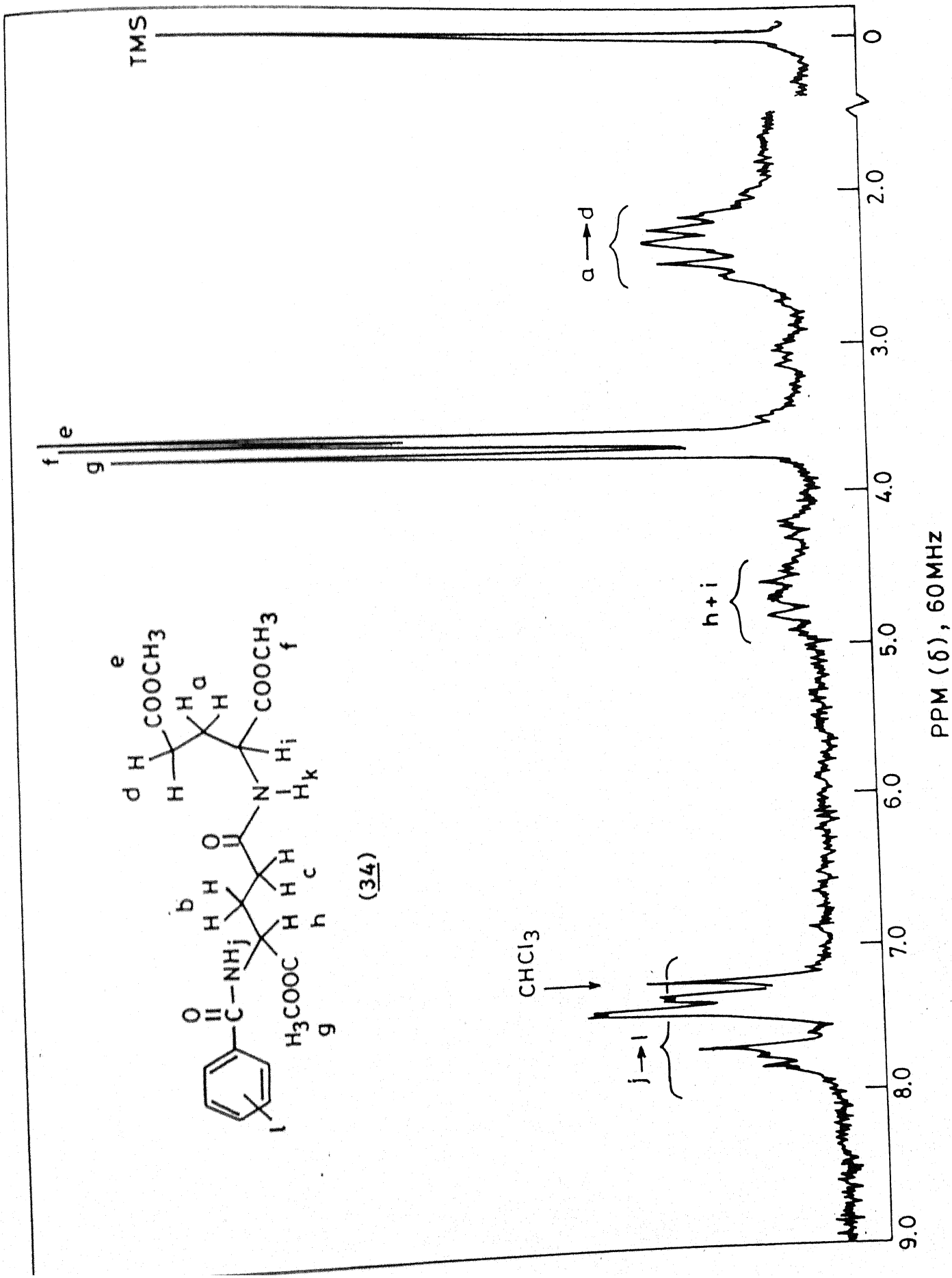
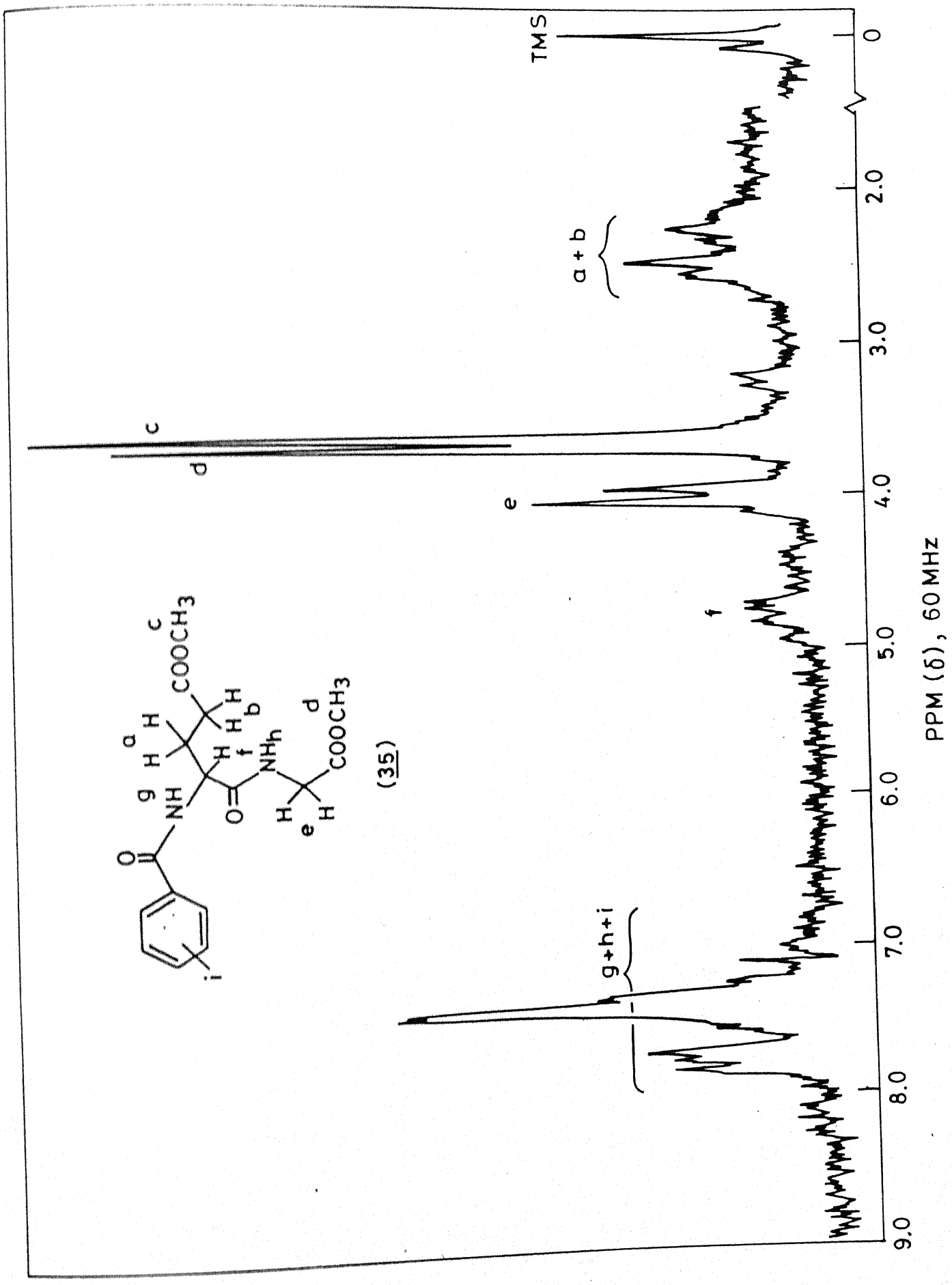

TMS
e
f
g
a — d
h+i
CHCl3
j
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
0
PPM (δ), 60 MHz
(33)



(34)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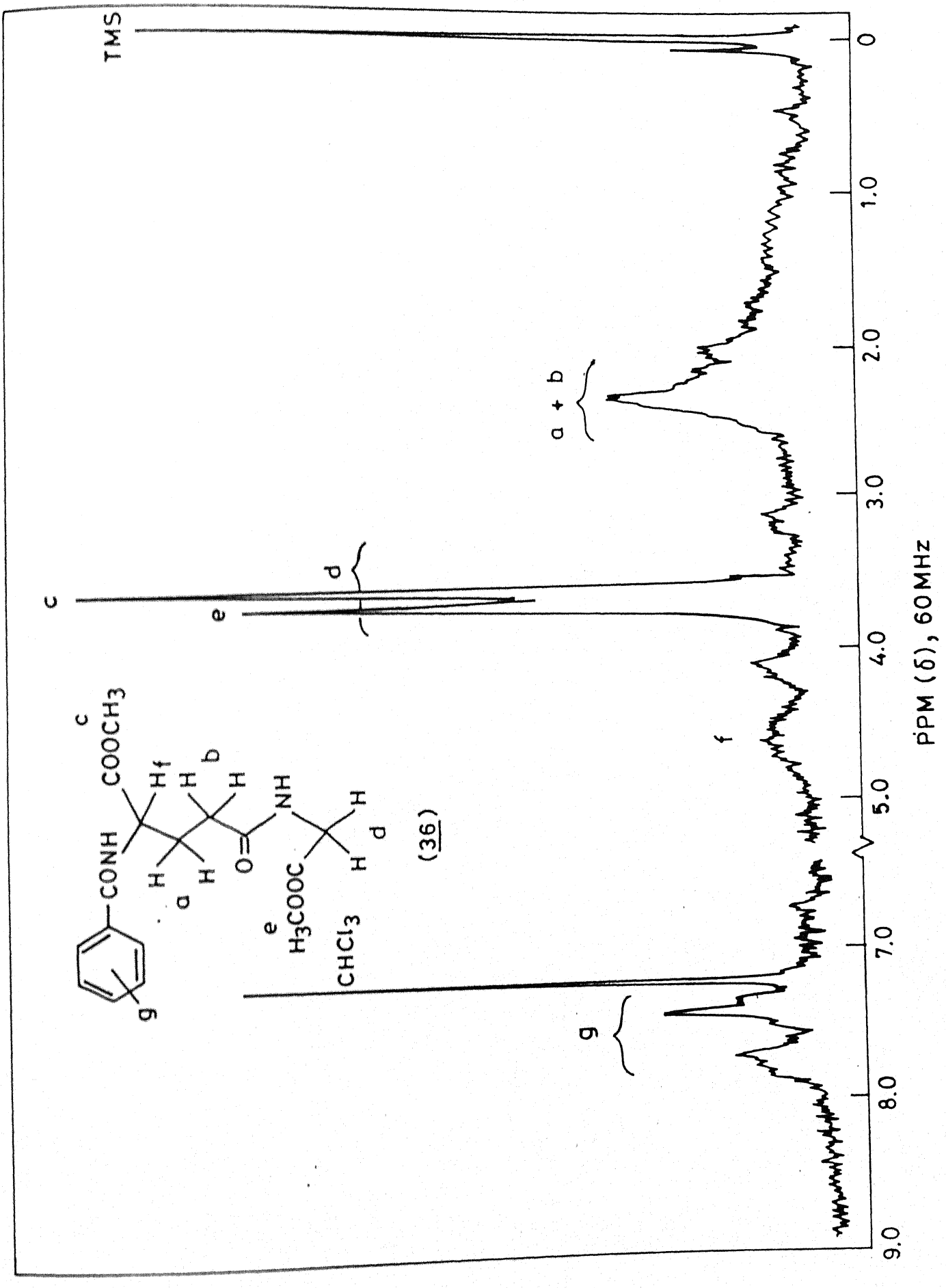
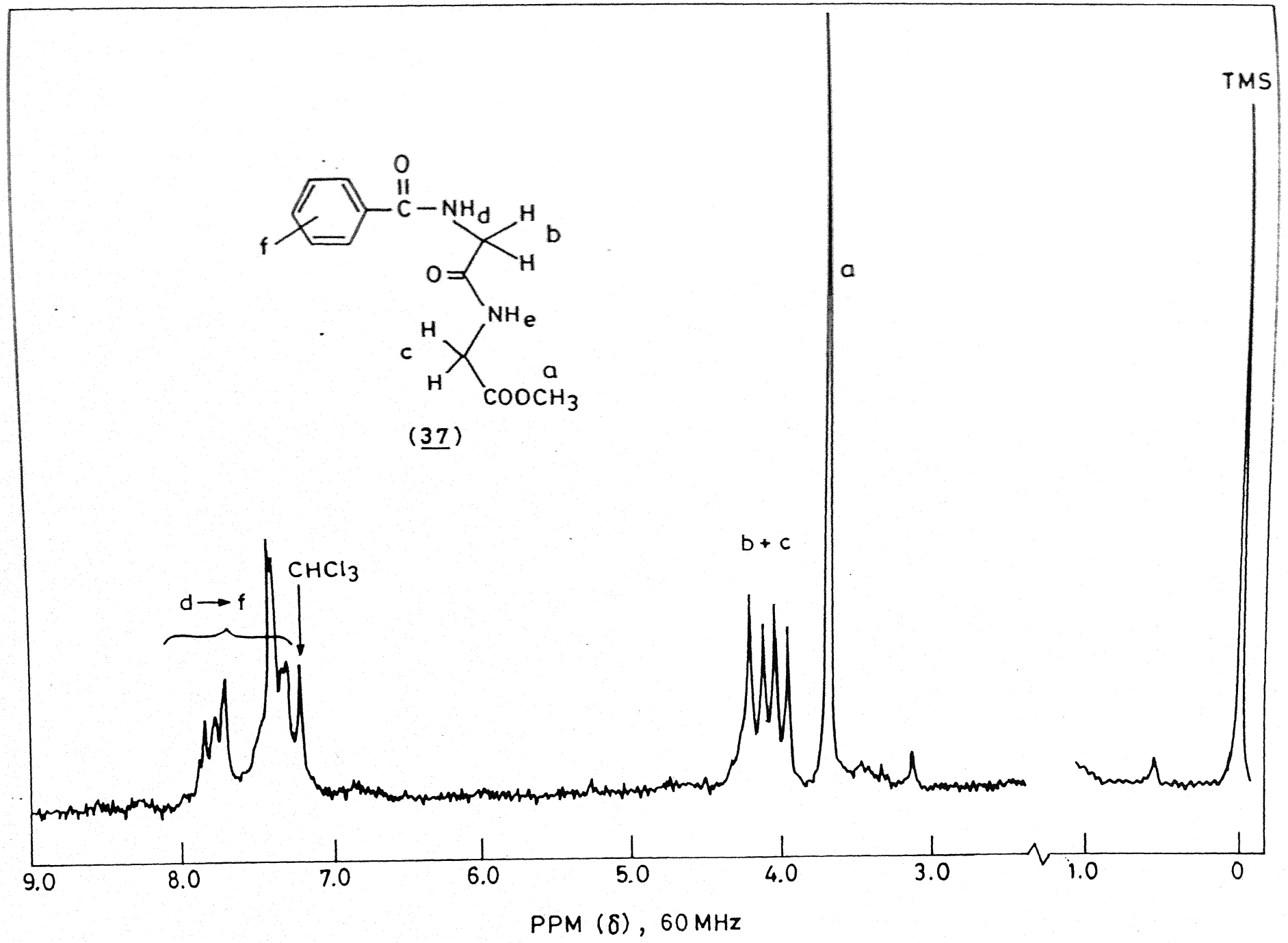
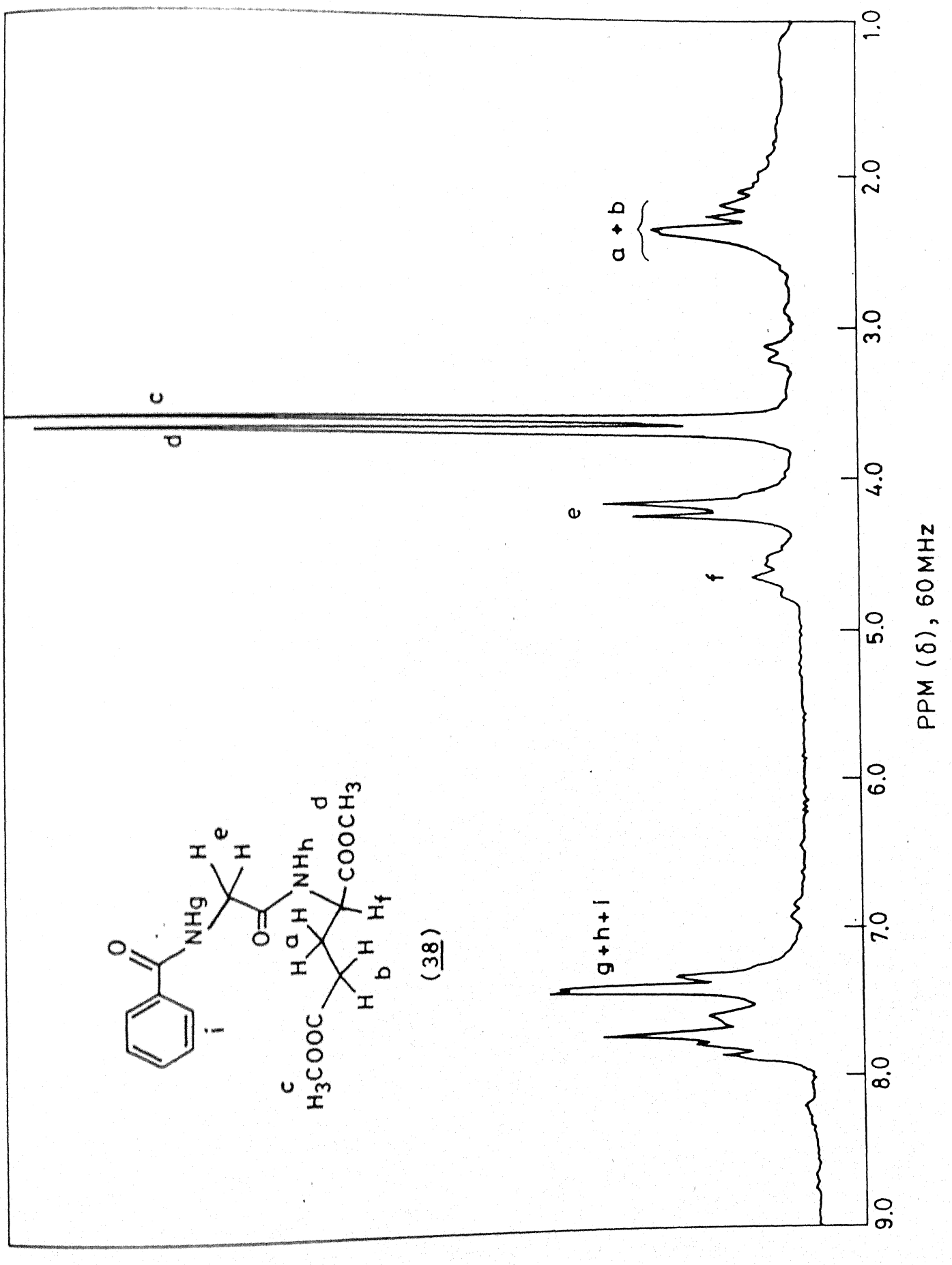
a+b
c
d
e
f
g+h+i
0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
PPM (δ), 60 MHz
COOCH3
COOCH3
(35)

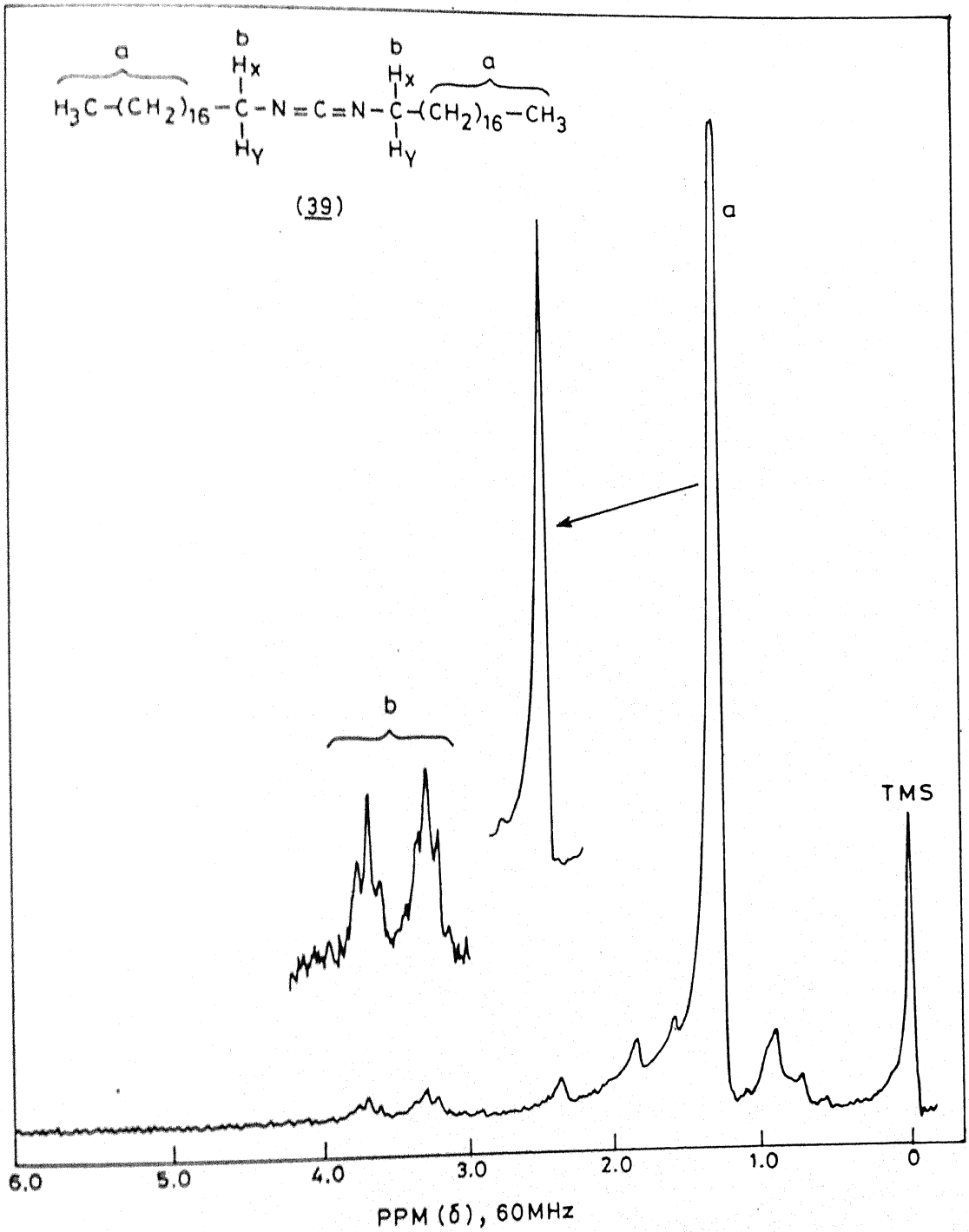

TMS
c
e
d
a + b
f
g
CONH
COOCH3
Hf
H
b
a
O=
NH
e
H3COOC
CHCl3
Hd
(36)
9.0
8.0
7.0
5.0
4.0
3.0
2.0
1.0
0
PPM (δ), 60MHz

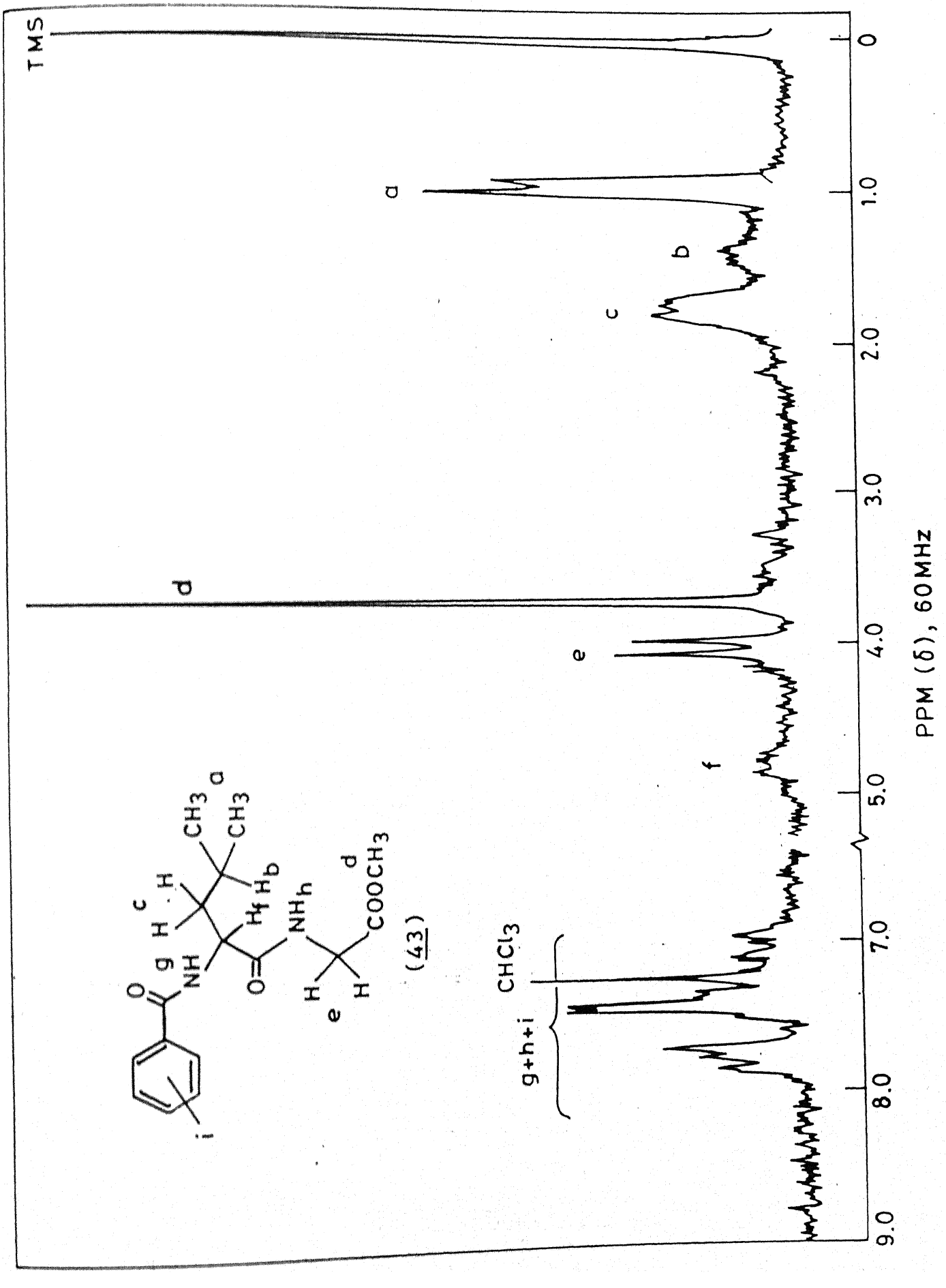


(37)

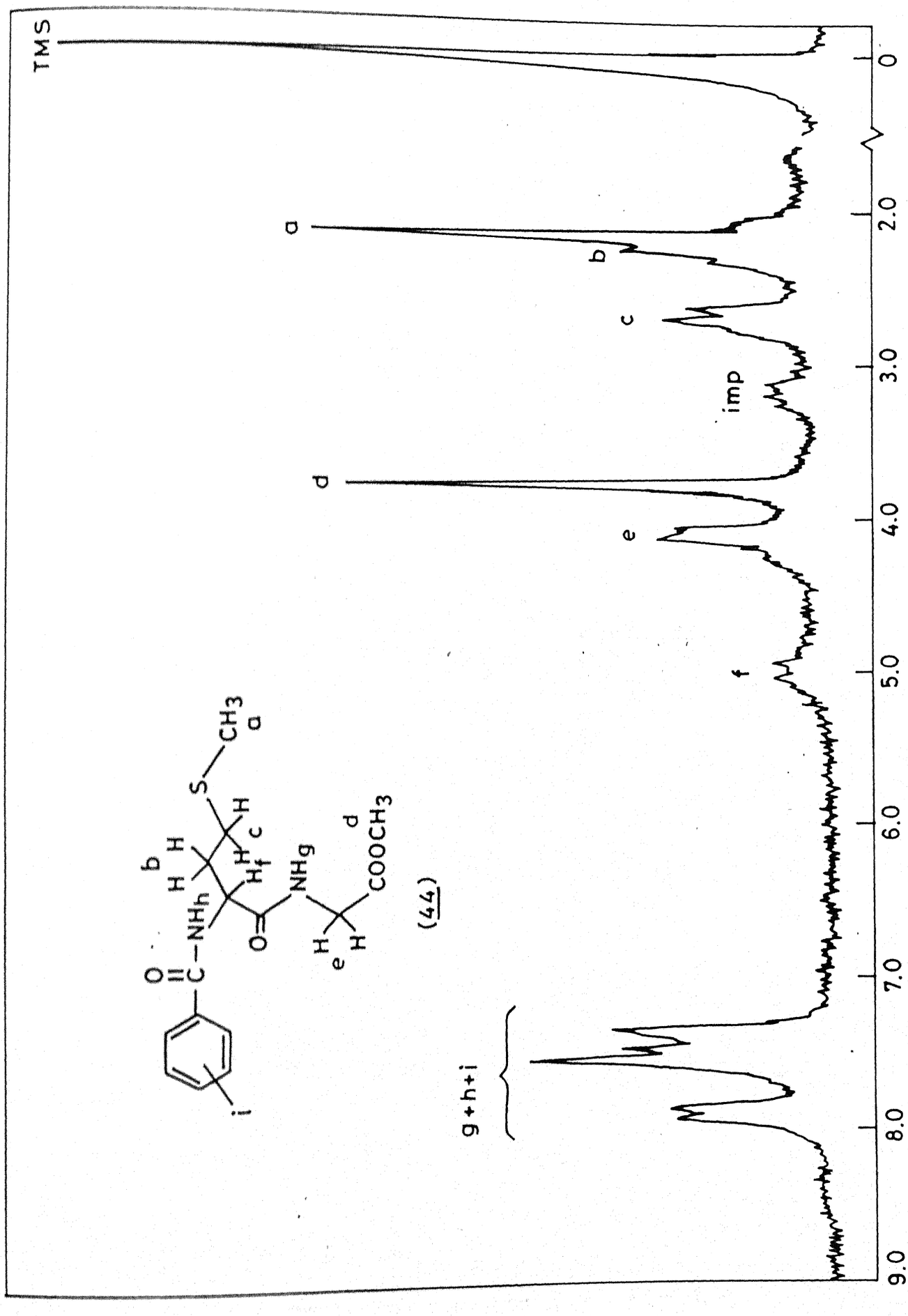


(38)

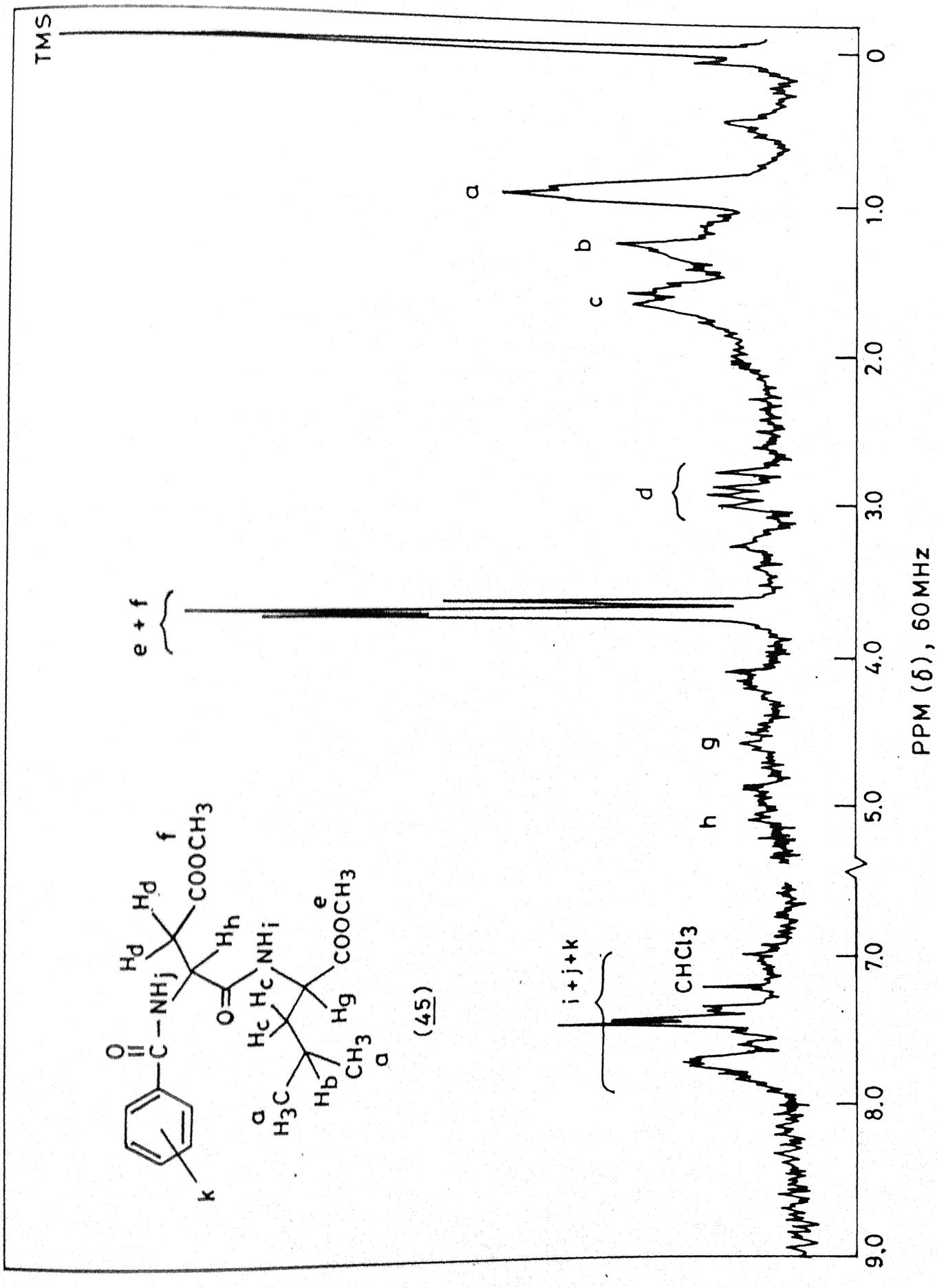

a
b
Hx
HY
b
Hx
HY
a
H3C-(CH2)16-C-N=C=N-C-(CH2)16-CH3
(39)
a
b
TMS
6.0
5.0
4.0
3.0
2.0
1.0
0
PPM (δ), 60MHz



(43)



(44)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a
b
c
d
e + f
h
g
i + j + k
CHCl3
0
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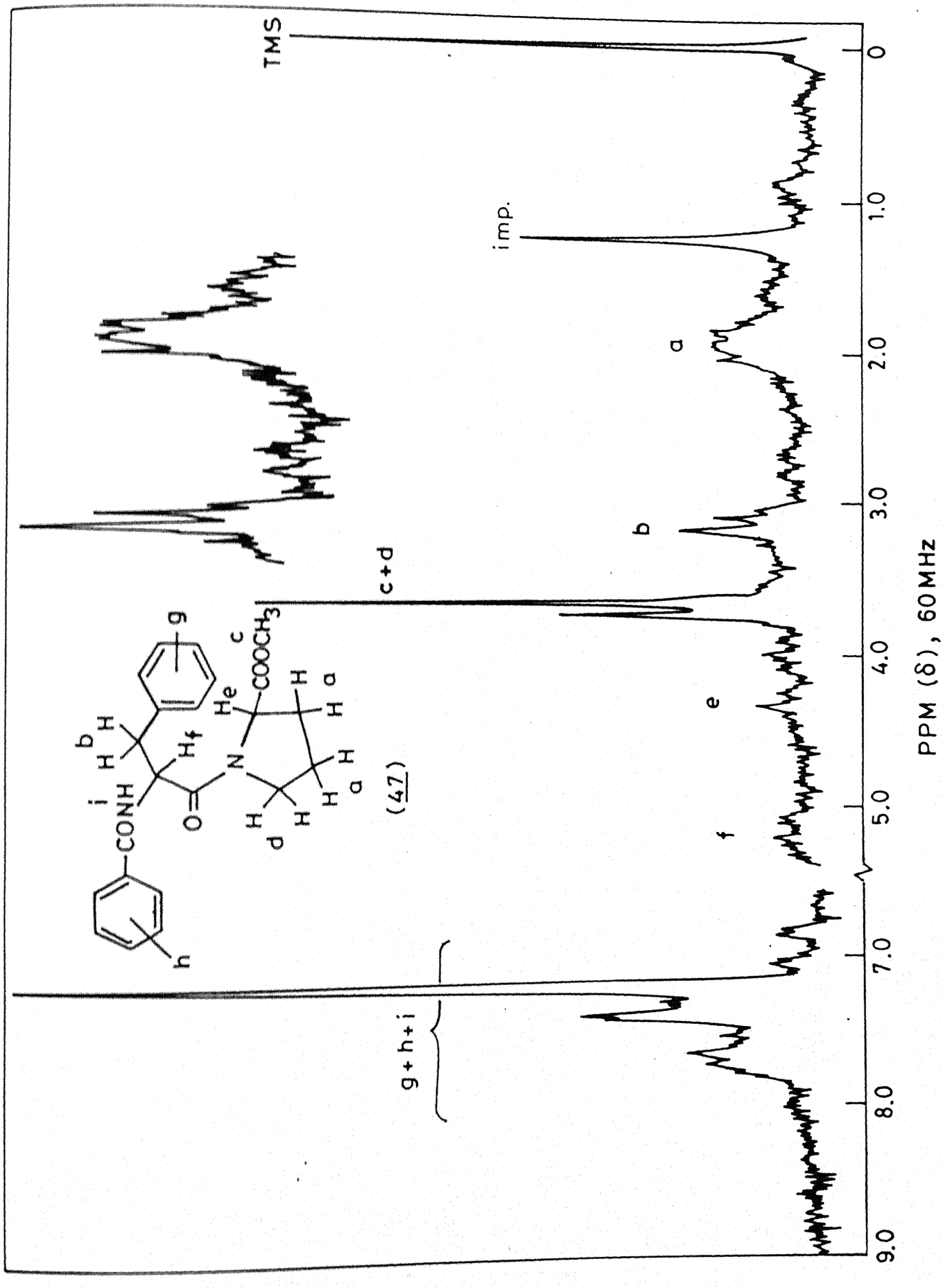
2.0
3.0
4.0
5.0
7.0
8.0
9.0
PPM (δ), 60 MHz
(45)


TMS
imp.
a
b
c+d
e
f
g+h+i
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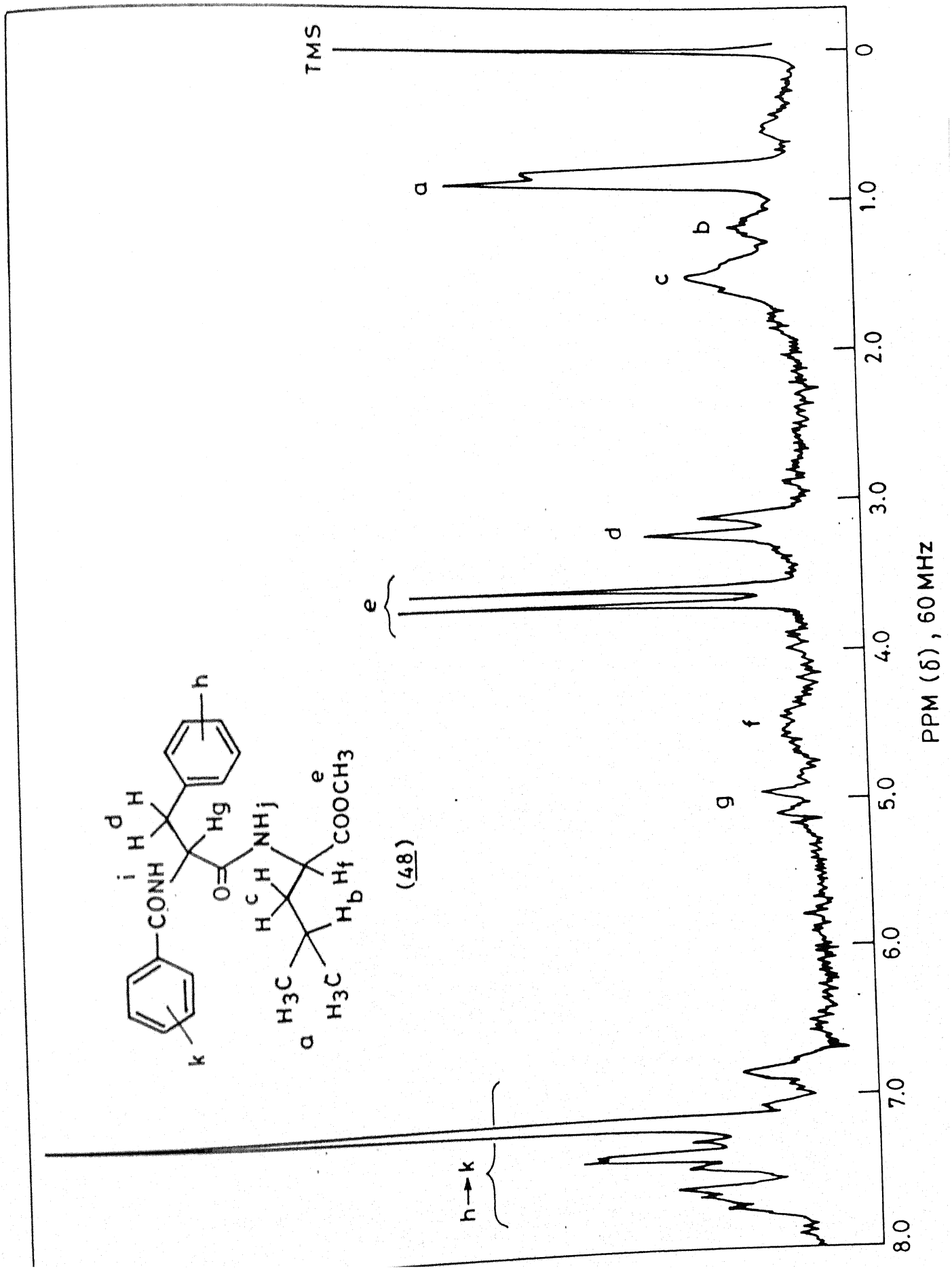
8.0
7.0
5.0
4.0
3.0
2.0
1.0
0
PPM (δ), 60MHz
(47)


TMS
a
b
c
d
e
f
g
h→k
PPM (δ), 60 MHz
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
(48)

E. EXPERIMENTAL

Melting points and boiling points are uncorrected. Infrared spectra were recorded on Perkin-Elmer Model 580 spectrophotometer either as neat liquids or as thin KBr wafers. NMR spectra were obtained on dilute solutions in $CDCl_3$ or CCl_4 or DMSO-d_6 on a Hitachi R600 (FT), E.M. 390 spectrometers. The chemical shifts are recorded in ppm with TMS at 0.00 as internal standard. Mass spectra were recorded with a Jeol instrument. HPLC analyses were carried out with Model 244 (Waters associates) using solvent system MeOH : H_2O (80:20) in reversed phase column. Elemental analyses were carried out in automatic C,H,N analysers. Silica gel G (MERCK) was used for tlc and column chromatography was done on silica gel (acme, 100-200 mesh) columns, which were invariably made from a slurry in benzene. Reactions were monitored, wherever possible by TLC. The organic extracts were invariably dried over anhydrous $MgSO_4$ and solvents evaporated in vacuo.

I. The Preparation of 1,4-DiMethyl2Tetrazoline-5-Thione (1) :

a. Methyl isothiocyanate :

To a vigorously stirred and ice salt cooled (-10 to -15°C) mixture of 10N-NaOH (90 ml) and CS_2 (55 ml, 900 mmol) was added, over 0.5 h, aq $MeNH_2$ (35%, 90 ml, 900 mmol). The stirred reaction mixture was warmed on a water bath for 1.5 h, the resulting bright red solution cooled to rt and admixed, over 1h, with $ClCO_2Et$ (88 ml, 900 mmol), left stirred for 0.5 h, the top layer of MeNCS separated, dried (Na_2SO_4) and distilled ; b.p. 118-122°C, yield 35 g (54%) (lit.[46]).

b. 1-Methyl-5-(methylthio)-1H-tetrazole (2) :

To refluxing NaN_3 (22.1 g, 340 mmol) in H_2O : EtOH :: 6:1 (35 ml) was added, under stirring, during 2 h, a solution of MeNCS (24.8 g, 340 mmol) in EtOH (20 ml). The reaction mixture was left stirred for 0.5 h, cooled to rt, admixed with, in drops, $(MeO)_2SO_2$ (32.2 ml, 340 mmol), was left stirred for 15 h, concentrated in vacuo, extracted with CH_2Cl_2 (6 x 30 ml), dried and distilled to give 36 g (83%) of (2) as an oil, bp 138-40°/10 torr. (lit.[47] bp 78°/ 10^{-3} torr.).

Anal. : Calcd. for $C_3H_6N_4S$ (mol.wt. 130) :
C, 27.60; H, 4.60; N, 43.07%
Found : C, 27.83 ; H, 4.91; N, 42.86%

nmr : δ (CCl_4) : 2.8 (s, 3H, SMe), 3.9 (s, 3H, NMe).

ms : m/z : 130 (M^+).

c. 1,4-Dimethyl-2-tetrazoline-5-thione (1) :

To stirred Dimethyl sulfate (10.5 ml, 110 mmol) held at 100° was added, in 0.5 h, (2) (14.5 g, 110 mmol). The mixture was left stirred for 0.25 h at 110°, cooled, admixed with dry MeCN (30 ml) followed by, in drops, dry NEt_3 (7.75 ml, 55 mmol), left stirred for 3 d, diluted with benzene (50 ml) and evaporated *in vacuo*. The benzene addition and evaporation was repeated thrice, the residue taken up in benzene (60 ml), left stirred for 15 h, decanted, the residue further stirred with additional three lots of benzene (3 x 25 ml), the combined extracts evaporated and the residue on crystallization from cyclohexane gave 6.5 g (45%) of (1), as colourless crystals, mp. 102-103°C (lit.[35] mp.107°C).

Anal : Calcd for $C_3H_6N_4S$(Mol.wt. 130).
C, 27.60 ; H, 4.60 ; N, 43.00%
Found C, 27.43 ; H, 4.90 ; N, 43.48%

ir : ν_{max}(KBr) cm^{-1} : 1360 (C=S).

nmr : δ ($CDCl_3$) : 3.9 (s, 6H, 2 x N -CH_3).

ms : m/z : 130 (M^+), 73 $(MeNCS)^+$.

II. The Reaction of 1-Methyl-5-(methylthio)-1H-tetrazole with Tosyl hydrazide : Isolation of 1,4-Dimethyl-2-tetrazoline-5-tosyl hydrazone (4) :

Dimethyl sulfate (1.9 ml, 20 mmol), was added, in drops, to (2) (2.6 g, 20 mmol) held at 120°C. The reaction mixture was cooled to rt, admixed with a solution of $TsNHNH_2$ (3.72 g, 20 mmol), in pyridine : NEt_3 :: 25 : 3

(28 ml), left stirred for 16 h, evaporated, the residue poured on to crushed ice, filtered, dried and crystallized from hot dioxane to give 0.75 g (14%) of (4), mp. 204-205°C.

anal : Calcd. for $C_{10}H_{14}N_6O_2S$ (Mol.wt. 282).
C, 42.56 ; H, 4.90 ; N, 29.8%
Found C, 42.86 ; H, 5.24 ; N, 29.34%

ir : ν_{max}(KBr) cm^{-1} : 3250 (-NH), 1620 (C=N), 1330, 1160 (SO_2).

nmr : δ($CDCl_3$) : 2.5 (s, 3H, CH_3)

ms : m/z : 282 (M^+), 197 (TsNHN $\equiv$CH $)^+$, 155 (TsH $)^+$.

III. The Reaction of 1-Methyl-5-(methylthio)-1H-tetrazole with Benzoyl hydrazide : Isolation of 1,4-Dimethyl-2-tetrazolinebenzoyl hydrazone (5a) :

Dimethyl sulfate (1.9 ml, 20 mmol) was added, in drops, to a solution of (2) (2.6 g, 20 mmol) in dry toluene (25 ml), held at 125°C. The reaction mixture was cooled, decanted, dried, admixed with a solution of $BzNHNH_2$ (2.72 g, 20 mmol) in pyridine : NEt_3 :: 25 : 3 (28 ml), left stirred for 16 h, evaporated. digested with crushed ice, filtered, dried and crystallized from acetone to give 2.4 g, (46%) of (5a), mp. 221-2°C. (lit.[47] mp. 222°).

anal : Calcd. for $C_{10}H_{12}N_6O$ (Mol.wt. 232).
C ; 46.50 ; H ; 5.17 ; N, 36.2%
Found C, 47.08 ; H, 5.62 ; N, 35.8%

ir : ν_{max}(KBr) cm^{-1} : 3240 (NH), 1645, 1545 (amide).

nmr : δ($CDCl_3$+ DMSO-d_6) : 3.69 (s, 6H, 2 x N-CH_3), 7.39-7.91 (m, 5H, aromatic).

IV. The Reaction of 1-Methyl-5-(methylthio)-1H-tetrazole with Ethyl carbazate : Isolation of 1,4-Dimethyl-2-tetrazoline-5-ethyl carbazone (5b) :

To compound (2) (3.9 g, 30 mmol) held at 125°C was added, in drops, $(MeO)_2SO_2$ (2.7 ml, 30 mmol). The reaction mixture was cooled to rt, admixed with a solution of NH_2NHCO_2Et (3.12 g, 30 mmol) in pyridine : NEt_3 :: 35 : 4.5 (39.5 ml), left stirred for 16 h, evaporated and the residue chromatographed on silica gel. Elution with benzene gave 0.8 g (20%) of (5b), mp. 120-124°C.

ir : ν_{max}(KBr) cm^{-1} : 3480 (NH), 1720, 1625 (urethane).

V. The Preparation of 1,4-Dimethyl-2-tetrazoline-5-hydrazone (6) :

a. By acidic hydrolysis of (5a) :

A solution of (5a) (1.16 g, 5 mmol) in 6NHCl (20 ml) was refluxed for 0.5 h, evaporated, the residue taken up in absolute ethanol and concentrated to give 0.2 g (25%) of (6), as hydrochloride, mp. 250-4°C. (lit.[47]).

nmr : $\delta(D_2O)$: 4.8 (s, 6H, 2 x N $-CH_3$).

b. By Saponification of (5b) :

To stirred and ice salt cooled 1N-NaOH (5 ml) was added, (5b) (0.2 g, 1 mmol). The reaction mixture was left stirred for 1 h, filtered, washed with cold water, and dried to give 0.06 g (46%) of (6), mp. 140°C.

ir : ν_{max}(KBr) cm^{-1} : 3260 (-NH).

VI. Oxidation of 1,4-Dimethyl-2-tetrazoline-(5)-hydrazone (6) with yellow mercuric oxide : Attempted preparation of 1,4-Dimethyl-2-tetrazoline-5-diazo-2-tetrazoline (7) :

A mixture of (6) (0.064 g, 0.5 mmol), yellow mercuric oxide (0.125 g,

0.58 mmol) anhydrous Na_2SO_4 (0.02 g), dry ether (2 ml) and ethanolic KOH (1 drop) was stirred for 0.5 h, filtered, and evaporated to give an intractable gum.

VII. Attempted Preparation Of The Carbene Dimer (9) : By thermolysis of (4) :

Thermolysis of (4) either neat or in xylene followed by analysis of the products failed to yield either the expected diazo compound (7) or any product possessing the N-Me grouping. The gummy residue obtained in each case exhibited the presence of the Ts moiety (nmr).

VIII. The Preparation of 1-Methyl- 2 -tetrazoline-5-thione (10) :

To a stirred solution of NaN_3 (9.74 g, 150 mmol) in water (200 ml) was added, in drops, MeNCS (7.30 g, 100 mmol). The mixture was refluxed for 8 h, cooled, extracted with ether, the aqueous layer cooled, adjusted to pH 3 with 2NHCl, extracted with ether (3 x 50 ml), dried, evaporated and the residue on crystallization from chloroform-hexane gave 5.4 g (46%) of (10) : mp. 125-127°C. (lit.[48] mp. 125-126°C).

ir : ν_{max}(KBr) cm^{-1} : 1330 (C=S)

nmr : δ ($CDCl_3$) : 4.0 (s, 3H, N-CH_3), 7.4 (br, 1H, NH).

ms : m/z : 116 (M^+), 73 ($MeNCS^+$).

IX. The Preparation of 1-Phenyl- 2 -tetrazoline-5-thione (11) :

A mixture of PhNCS (8.10 g, 60 mmol), NaN_3 (5.85 g, 90 mmol) and water (60 ml) was refluxed for 6 h, extracted with ether (2 x 50 ml), the aqueous layer adjusted to pH 3 with 2NHCl, filtered, washed with cold water,

dried and crystallized from EtOH to give 0.8 g (84%) of (11), mp. 154°C (lit.[48] mp. 150°C).

ir : ν_{max}(KBr) cm^{-1} : 3500 (br, NH), 1360 (C=S)

nmr : $\delta(CDCl_3)$: 7.6 (d, t, 3H, o,p-protons), 8.05 (m, 2H, m-protons).

X. The Preparation of 1-Phenyl-4-(hydroxymethyl)-tetrazoline-5-thione (12) :

Formalin (35%, 1.5 ml, 20 mmol) was added, in drops, to (11) (0.22g, 1.2 mmol). The mixture was refluxed for 0.5 h, cooled and filtered to give 0.24 g (93%) of (12) mp. 94°C (lit.[38] mp. 97-98°C).

anal : Calcd. for $C_8H_8N_4S$ (Mol.wt. 208).
C, 46.15 ; H, 3.84 ; N, 26.92%
Found C, 45.78 ; H, 4.35 ; N, 26.79%

ir : ν_{max}(KBr) cm^{-1} : 3300 (OH), 1360 (C=S)

nmr : $\delta(CDCl_3)$: 4.9 (OH), 5.8 (s, 2H, N-CH_2), 7.5 (d, t, 3H, o,p-protons), 8.0 (q, 2H, m-protons).

XI. The Preparation of the Mannich base 1-Methyl-4-(piperidino methyl)-tetrazoline-5-thione (13) :

A solution of (10) (0.58 g, 5 mmol) in methanol (5 ml) was admixed with formalin (35%, 0.57 ml, 6.6 mmol) to which piperidine (0.55 ml, 5 mmol) had been added, left aside for 2 h, evaporated and distilled to give 0.6 g (56%) of (13).

ir : ν_{max}(KBr) cm^{-1} : 1340 (C=S)

nmr : $\delta(CDCl_3)$: 1.55 (m, 6H, $(CH_2)_3$), 2.7 (m, 4H, N-$(CH_2)_2$), 3.9 (s, 3H,

$N-CH_3$), 5.2 (s, 2H, NCH_2-N).

ms : m/z : 214 MH^+, 154 (MeN=C=NCH_2-NHpip.)$^+$, 101 (Me-N(CS)N=CH_2)$^+$.

XII. Attempted Preparation of the Mannich base, 1-Phenyl-4-(N,N-diethylamino-methyl)-tetrazoline-5-thione : Isolation of 1-Phenyl-5-(diethylamino) tetrazoline(14) :

a. From 1-Phenyl- 2 -tetrazoline-5-thione (11) :

To a solution of (11) (0.89 g, 5 mmol) in methanol (5 ml) admixed with formalin (35%, 0.57 ml, 6.6 mmol) was added, in drops, Et_2NH (0.56 ml, 5.6 mmol). The reaction mixture was left aside for 2 h and filtered to give 0.648 (58%) of (14), mp. 111°C. (lit.[38] mp. 110-111°).

nmr : $\delta(CDCl_3)$: 1.4 (t, 6H, $CH_2(CH_3)_2$), 3.15 (q, 4H, $N(CH_2)_2$-CH_3), 7.5 (d, t, 3H, o,p-protons), 8.0 (m, 2H, m-protons).

b. From 1-Phenyl-4(hydroxymethyl)-tetrazoline-5-thione (12) :

To neat (12) (0.208 g, 1 mmol), was added Et_2NH (0.12 ml, 1.2 mmol) in drops. The reaction mixture was cooled and filtered to give 0.085 (39%) of (14), mp. 110-111°C (lit.[38] mp. 110°C).

XIII. The Preparation of the Mannich base 1-Phenyl-4(morpholinomethyl)-tetrazoline--5-thione (15) :

a. From 1-Phenyl- 2 -tetrazoline-5-thione (11) :

A solution of (11) (0.178 g, 1 mmol) in methanol (1 ml), was admixed with formalin (35%, 0.12 ml, 1.2 mmol) - to which morpholine (0.1 ml, 1.2 mmol) had been added - left aside for 2 h, filtered and crystallized from EtOAc-hexane

to give 0.188 g (67%) of (15) mp. 154°C. (lit.[38] mp. 154°C).

ir : ν_{max}(KBr) cm^{-1} : 1350 (C=S)

nmr : $\delta(CDCl_3)$: 2.8 (t, 4H, $N(\underline{CH_2})_2$), 3.7 (t, 4H, $O(\underline{CH_2})_2$), 5.3 (s, 2H, $N\text{-}\underline{CH_2}\text{-}N$), 7.5 (d, t, 3H, o,p-protons), 7.9 (m, 2H, m-protons).

b. From 4(Hydroxymethyl-1-phenyl-tetrazoline-5-thione (12) :

To neat (12) (0.208 g, 1 mmol), was added morpholine (0.1 ml, 1.2 mmol), cooled and crystallized from EtOAc-hexane to give 0.208 g (75%) of (15) mp.152-3°C.

XIV. The Preparation of the Mannich base 1-Phenyl-4(piperidinomethyl) tetrazoline--5-thione (16) :

a. From 1-Phenyl-2 -tetrazoline-5-thione(11) :

To a solution of (11) (0.89 g, 5 mmol) was added formalin : piperidine :: 0.6 ml (35%, 6.6 ml) : 0.55 ml (5 mmol). The reaction mixture was left aside for 2h, filtered and dried to give 0.11 g (74%) of (16), mp. 136-137°C. (lit.[38] mp.136°C).

ir : ν_{max}(KBr) cm^{-1} : 1350 (C=S)

nmr : $\delta(CDCl_3)$: 1.5 (m, 6H, $(\underline{CH_2})_3$), 2.3 (m, 4H, $N(\underline{CH_2})_2$), 5.3 (s, 2H, $N\text{-}\underline{CH_2}\text{-}N$), 7.5 (d, t, 3H, o,p-protons), 8.0 (m, 2H, m-protons).

b. From 1-Phenyl-4(hydroxymethyl)-tetrazoline-5-thione (12) :

To neat (12) (0.208 g, 1 mmol) was added piperidine (0.12 ml, 1.1 mmol), cooled and pressed dry to give 0.25 g (90%) of (16), mp. 134°C. (lit.[38] mp. 136°C).

XV. The Preparation of 1-Methyl-5(benzylthio)-1H-tetrazole (17) :

To a solution of (10) (0.116 g, 1 mmol) in dry benzene (5 ml) admixed

with NEt_3 (0.2 ml, 1.5 mmol) was added, in drops, $PhCH_2Br$ (0.257 g, 1.5 mmol). The reaction mixture was left stirred for 4 h and evaporated to give 0.13 g (63%) of (17) as an oil, bp. 180°C/2 torr.

nmr : δ ($CDCl_3$) : 3.75 (s, 3H, N-CH_3), 4.5 (s, 2H, S-CH_2), 7.3 (s, 5H, aromatic).

ms : m/z : 206 (M^+).

XVI. The Preparation of 1-Benzyl-2 -tetrazoline-5-thione (18) :

A mixture of benzylisothiocyanate (7.5 g, 50 mmol) NaN_3 (4.87 g, 75 mmol) and water (100 ml) was refluxed for 8 h, extracted with ether (2 x 50 ml), the aqueous layer adjusted to pH 3 with 2N-HCl, extracted with ether (3 x 50 ml), dried and crystallized from EtOAc-hexane to give 5.45 g (56%) of (18), mp. 143°C. (lit.[48] mp. 144°C).

ir : ν_{max} (KBr) cm^{-1} : 3090 (NH), 1360 (C=S).

nmr : δ ($CDCl_3$) : 5.4 (s, 2H, NCH_2-Ph), 7.4 (br, 5H, Ph).

XVII. The Preparation of 1-Benzyl-5(benzylthio)-1H-tetrazole (19) :

To a solution of (19) (0.96 g, 5 mmol) in dry ether (10 ml) admixed with NEt_3 (0.66 ml, 5 mmol) was added, in drops, $C_6H_5CH_2Cl$ (0.63 g, 5 mmol). The reaction mixture was refluxed for 19 h, NEt_3HCl was filtered off, evaporated and the residue on crystallization from EtOAc-hexane gave 0.9 g (64%) of (19), mp. 59-60°C. (lit.[39] mp. 62.5-63.5°C).

ir : ν_{max} (KBr) cm^{-1} : 3000 (NH), 1610 (C=N), 1505 (N=N).

nmr : $\delta(CDCl_3)$: 4.5 (s, 2H, S-$\underline{CH_2}$-PH), 5.3 (s, 2H, NC$\underline{H_2}$-Ph), 7.3 (br, 10H, aromatic).

XVIII. The Preparation of 1-Phenyl-5(thioacetate)-1H-tetrazole (20) :

To a solution of (11) (0.356 g, 2 mmol) in dry benzene (10 ml) admixed with NEt_3 (0.26 ml, 2 mmol) was added, in drops, $BrCH_2CO_2Et$: dry benzene :: 0.5 g (3 mmol) : 5 ml. The reaction mixture was left stirred for 10 h, NEt_3HBr was filtered off, evaporated and the residue on crystallization from benzene : hexane to give 0.19 g (38%) of (20), mp. 84-6°C. (lit.[49] mp. 87-88°).

anal : Calcd. for $C_{11}H_{12}N_4O_2$

C, 50.00 ; H, 4.54 ; N, 21.21%

Found C, 49.41 ; H, 4.40 ; N, 20.87%

ir : ν_{max}(KBr) cm^{-1} : 1740 (ester)

nmr : $\delta(CDCl_3)$: 1.3 (t, 3H, O-CH_2-$\underline{CH_3}$), 4.25 (s, 2H, S-$\underline{CH_2}$), 4.3 (q, 2H, O-$\underline{CH_2}$-CH_3), 7.6 (s, 5H, aromatic).

XIX. The Preparation of 1-Phenyl-5(thioacetic acid)-1H-tetrazole (21) :

a. From 1-Phenyl-2-tetrazoline-5-thione (11) :

To a solution of (11) (0.356 g, 2 mmol) in dry benzene (10 ml) admixed with NEt_3 (0.26 ml, 2 mmol) was added in drops, $BrCH_2CO_2H$: dry benzene :: 0.42 g (3 mmol) : 5 ml. The reaction mixture was left stirred for 18 h, NEt_3HBr was filtered off, and evaporated to give 0.124 g (30%) of (21) as an oil.

b. By saponification of (20) :

To stirred and ice salt cooled 1N KOH (5 ml) was added, (20) (0.264 g, 1 mmol). The reaction mixture was left stirred overnight, cooled to 0°C, adjusted to pH 3, extracted with ether (2 x 50 ml), dried and evaporated to give 0.11 g (46%) of (21), as an oil.

ir : ν_{max}(neat) cm^{-1}: 3700-2300 (br), 1710 (COOH)

nmr : δ ($CDCl_3$) : 4.2, 4.3 (2H, $C\underline{H}_2$-COOH), 7.32-8.2 (m, 5H, aromatic).

ms : m/z : 237 MH^+.

XX. The Preparation of 1-Cyclohexyl-3-(3-dimethylaminopropyl)carbodiimide metho-p-toluenesulfonate (22) :

a. Cyclohexyl isothiocyanate :

To a vigorously stirred and ice-cooled (0°C) solution of cyclohexylamine (28.73 g, 260 mmol) in dry ether (200 ml) was added, in drops, CS_2 (10.07 g, 130 mmol). The resulting solid was filtered, treated with mercuric chloride (35 g, 130 mmol) in water (375 ml) at 90°C for 0.25 h and steam distilled to give 14.0g (76%) of cyclohexyl isothiocyanate. (lit.[50]).

ir : ν_{max}(neat) cm^{-1} : 2136 (isothiocyanate).

b. 1-Cyclohexyl-3-(3-dimethylaminopropyl)thiourea (23) :

To a stirred and ice cooled solution of cyclohexyl isothiocyanate (4.512 g, 32 mmol) in dry ether (20 ml) was added, in drops, N,N-dimethyl-1,3-propanediamine (3.264 g, 32 mmol) in ether (20 ml). The reaction mixture was

left stirred for 2 h, filtered and dried to give 6.0 g (78%) of (23), mp. 70°C. (lit.[21] mp. 71°C).

anal : Calcd. for $C_{12}H_{25}N_3S$(Mol.wt. 243)
C, 59.20 ; H, 10.37 ; N, 17.25%
Found : C, 59.16 ; H, 10.35 ; N, 17.36%

ir : ν_{max}(KBr) cm^{-1} : 3240 (NH), 1650 (thiourea)

c. 1-Cyclohexyl-3-(3-dimethylaminopropyl)carbodiimide (24) :

To vigorously stirred (23) (0.243 g, 20 mmol) in dry CH_2Cl_2 (30 ml) was added yellow HgO (10.8 g, 50 mmol). The reaction mixture was left stirred for 20 h, admixed with another portion of yellow HgO (2.12 g, 10 mmol) and left stirred for additional 5 h. The reaction mixture was filtered and evaporated to give 1.6 g (76%) of (24) as a thick syrup.(lit.[21]).

anal : Calcd. for $C_{12}H_{23}N_3$(Mol.wt. 209)
C, 68.82 ; H, 11.08 ; N, 20.08%
Found : C, 68.76 ; H, 10.96 ; N, 19.96%

ir : ν_{max}(neat) cm^{-1} : 2160 (carbodiimide)

d. 1-Cyclohexyl-3-(3-dimethylaminopropyl)carbodiimide metho-p-toluene-sulphonate (22) :

A mixture of methyl-p-toluenesulphonate[51](2.0 g, 11 mmol) and (24) (2.29 g, 11 mmol) in dry ether (40 ml) was left stirred for 24 h, filtered and dried to give 2.5 g (60%) of (22), mp. 157-8°C. (lit.[21] mp. 164.4-165.4°C).

ir : ν_{max}(KBr) cm^{-1} : 2140 (carbodimide)

nmr : δ (D_2O) : 1.4 (br, 12H, $(\underline{CH_2})_5$-CH_2-$\underline{CH_2}$-CH_2), 2.3 (s, 3H, $\underline{CH_3}$), 2.92-3.6 (br, 14H, $\underline{Me_3}\overset{+}{N}$-$\underline{CH_2}$, NCH_2 and =N-$\underline{CH}$), 7.4 (d, d, 4H, aromatic).

XXI. 1-Cyclohexyl-3-(3-diethylaminopropyl)carbodiimide - etho-p-toluenesulphonate (25) :

a. 1-Cyclohexyl-3-(3-diethylaminopropyl)thiourea (26) :

To a stirred and ice cooled solution of cyclohexyl isothiocyanate (2.82 g, 20 mmol) in dry ether was added, in drops a solution of N,N-diethylamino-propylamine (2.6 g, 20 mmol) in dry ether (15 ml). The reaction mixture was left stirred for 2 h, filtered and dried to give 4.8 g (88%) of (26), mp. 74°C. (lit.[52] mp. 76-78°C).

anal : Calcd. for $C_{14}H_{29}N_3S$(Mol.wt. 271)
C, 61.99 ; H, 10.70 ; N, 15.49%
Found : C, 61.76 ; H, 9.86 ; N, 15.12%

ir : ν_{max}(KBr) cm^{-1} : 3210 (NH).

nmr : δ($CDCl_3$): 0.9 (t, 6H, N-($CH_2\underline{CH_3}$)$_2$, 2.5 (m, 6H, $\underline{H_2}C$-N-($\underline{CH_2}CH_3$)$_2$), 3.5 (m, 3H, $\underline{CH_2}NHC(S)NH\underline{CH}$).

ms : m/z: 271(MH^+), 199 $(M+H-NHEt_2)^+$.

b. 1-Cyclohexyl-3-(3-diethylaminopropyl)carbodiimide (27) :

To a vigorously stirred mixture of (26) (5.42 g, 40 mmol) in dry CH_2Cl_2 (60 ml) was added yellow HgO (21.6 g, 100 mmol). The reaction mixture

was left stirred for 20 h admixed with another portion of yellow HgO (4.24g, 20 mmol) left stirred for 5 h, filtered and evaporated to give 3.8 g (80%) of (27) as a thick syrup.[52]

ir : ν_{max}(neat) cm^{-1} : 2125 (carbodiimide)

nmr : $\delta(CDCl_3)$: 1.0 (t, 6H, N-($CH_2\underline{CH_3}$)$_2$), 2.5 (m, 6H, $\underline{H_2}$C-N-($\underline{CH_2}CH_3$)$_2$), 3.25 (t, 2H, -$\underline{H_2}$C-N=), 3.5 (br, 1H,=N-CH).

ms : 256 (M H^++H_2O), 183 (M H^++H_2O-Et_2NH).

c. 1-Cyclohexyl-3-(3-diethylaminopropyl)carbodiimide etho-p-toluenesulphonate (25) :

A mixture of Ethyl-p-toluenesulphonate (1.0 g, 5 mmol) and (27) (1.185 g, 5 mmol) in dry ether (25 ml) was left stirred for 24 h, filtered and dried to give 1.18 g (54%) of (25), mp. 148-50°C.

ir : ν_{max}(KBr) cm^{-1} : 2145 (carbodiimide)

XXII. The Preparation of Phthaloyl Gly-Gly-OMe (28) :

a. Phthaloyl-Gly :

A mixture of Glycine (7.5 g, 100 mmol) and finely ground Phthalic anhydride (14.9 g, 100 mmol) was held at 150°C for 0.5 h. The reaction mixture was cooled and crystallized from methanol-water (1:1) to give 17.1 g (83%) of Phthaloyl Gly, mp. 193°C. (lit.[53] mp. 192°).

nmr : $\delta(CDCl_3)$: 4.5 (s, 2H, $\underline{CH_2}$), 2.9 (br, 2H, $\underline{NH_2}$), 7.8 (d, d, 4H, aromatic).

b. Gly-OMe :

To an ice-cooled and stirred suspension of Gly-OMeHCl (0.625 g, 5 mmol) in dry CH_2Cl_2 (25 ml) was added, in drops, NEt_3 (0.85 ml, 6 mmol). The reaction mixture was left aside for 0.5 h, poured on to water, the organic layer dried and evaporated to give 0.340 g (76%) of Gly-OMe which was used without delay.

c. Phthaloyl-Gly-Gly-OMe (28) :

A mixture of Phthaloyl Gly (0.615 g, 3 mmol) Gly-OMe (0.265 g, 3 mmol), the water soluble carbodiimide (25) (1.311 g, 3 mmol) and H_2O (20 ml) was left stirred for 6 h, filtered and crystallized from EtOAc-hexane to give 0.58 g (83%) of (28), mp. 194°C.

ir : ν_{max}(KBr) cm^{-1} : 1770, 1660 (Phth), 1735 (Ester).

nmr : $\delta(CDCl_3)$: 3.7 (s, 3H, $COOC\underline{H}_3$), 4.0 (d, 2H, $NH\text{-}C\underline{H}_2COOCH_3$), 4.4 (s, 2H, $NC\underline{H}_2\text{-}COOCH_3$), 6.4 (br, 1H, NH), 7.7 (m, 4H, aromatic).

ms : m/z : 276 (M^+).

XXIII. The Preparation of Phthaloyl-Phe-Leu-OMe (29) :

a. Phthaloyl-L-Phenylalanine :

A mixture of L-phenylalanine (4.95 g, 30 mmol) and phthalic anhydride (4.47 g, 30 mmol) was held at 150°C for 0.5 h. The reaction mixture was cooled and crystallized from methanol-water (1:1) to give 7.2 g (86%) of Phthaloyl-Phe, mp. 184°C.(lit. [54] mp. 185°).

ir : ν_{max}(KBr) cm^{-1}: 1740 (COOH), 1660 (Phth).

nmr : $\delta(CDCl_3)$: 3.5 (m, 2H, $C\underline{H}_2$-Ph), 5.0 (m, 1H, tert.proton), 7.1 (s, 5H, Ph), 7.7 (s, 4H, Phth).

b. Leu-OMe :

To an ice-cooled and stirred suspension of Leu-OMe.HCl (1.086 g, 6 mmol) in dry CH_2Cl_2 (30 ml), was added, in drops, NEt_3 (1.03 ml, 7.2 mmol), the mixture left aside for 0.5 h, poured on to water, the organic layer separated, dried and evaporated to give 0.400 g (50%) of Leu-OMe which was used without delay.

c. Phthaloyl-Phe-Leu-OMe (29) :

A mixture of Phthaloyl-Phe (0.59 g, 2 mmol), Leu-OMe (0.290 g, 2 mmol), water soluble carbodiimide (25) (0.874 g, 2 mmol) and H_2O (15 ml) was left stirred for 6 h, filtered and crystallized from EtOAc-hexane to give 0.37 g (46%) of (29), mp. 112-115°C.

ir : ν_{max}(KBr) cm^{-1} : 1780, 1660 (Phth), 1740 (Ester).

nmr : $\delta(CDCl_3)$: 1.0 (d, 6H, -CH-($C\underline{H}_3$)$_2$), 1.65 (m, 3H, $CHC\underline{H}_2C\underline{H}Me_2$), 3.6 (d, 2H, $C\underline{H}_2$Ph), 3.7 (s, 3H, COO$\underline{Me}$), 4.6 (m, 1H, CONH-$C\underline{H}$-), 5.2 (t, 1H, N-$C\underline{H}$-CH_2), 6.7 (m, 1H, NH), 7.15 (s, 5H, Ph), 7.7 (s, 4H, Phth).

XXIV. The Preparation of Amino Acid Esters :

a. Glycine Methyl Ester HCl (Gly-OMe.HCl) :

Dry HCl was passed through a solution of Glycine (10 g, 130 mmol) in absolute methanol (150 ml) for 0.5 h, concentrated to 20-30 ml and crystallized

from dry methanol-ether to give 11.4 g (68%) of Gly-OMe.HCl, mp. 174°C. (lit.[55] mp. 175).

ir : ν_{max}(KBr) cm^{-1} : 2980 (br, salt), 1740 (ester), 1565 (amide).

b. L-Leucine Methyl Ester HCl (Leu-OMe.HCl) :

Dry HCl was passed through a stirred suspension of Leucine (5 g, 38 mmol) in dry methanol (30 ml) for 2 h. The resulting clear solution was evaporated in vacuo, the residue dissolved in minimum amount of dry methanol, admixed with dry ether, refrigerated overnight, filtered and dried in vacuo to give 5.0 g (71%) of Leu-OMe.HCl. mp. 146°C(lit.[56] mp. 151°C).

ir : ν_{max}(KBr) cm^{-1} : 3400 (br, salt), 1730 (ester).

nmr : $\delta(D_2O)$: 1.0 (d, 6H, $-CH(C\underline{H}_3)_2$), 1.5 (br, 1H, $C\underline{H}-(CH_3)_2$), 1.7 (br, 2H, $CH-C\underline{H}_2-CH$), 3.8 (s, 3H, $COOC\underline{H}_3$), 4.2 (q, 1H, $C\underline{H}-CH_2$).

c. Phenylalanine Methyl Ester Hydrochloride (Phe-OMe.HCl) :

Thionyl chloride (4.9 ml, 67 mmol), in drops, followed by L-Phenylalanine (9 g, 54.5 mmol) was added to stirred and ice-cooled dry methanol (45 ml). The mixture was allowed to attain rt, refluxed for 2 h, the clear solution evaporated and the residue on crystallization from dry methanol - dry ether gave 10.5 g (89%) of Phe-OMe.HCl as white needles, mp. 161°C. (lit.[57] mp. 160°C).

ir : ν_{max}(KBr) cm^{-1} : 3320 (salt), 1750 (ester).

d. Phe-OMe :

Under conditions described in EXPERIMENT XXII b, Phe-OMe.HCl (2.15 g, 10 mmol) and NEt_3 (1.8 ml, 13 mmol) gave 0.96 g (54%) of Phe-OMe as an oil, which was used without delay.

e. Proline-Methyl Ester HCl (Pro-OMe.HCl) :

Dry HCl was passed through a stirred solution of Proline (5.75 g, 50 mmol) in dry methanol (60 ml) for 6 h. The resulting clear solution was evaporated in vacuo, the residue allowed to stand in a desiccator over P_2O_5 and NaOH overnight to give 5.18 g (63%) of Pro-OMe.HCl as thick syrup.[58]

ir : ν_{max}(neat) cm^{-1} : 2830 (br), 1735 (ester).

f. Pro-OMe :

Under conditions described in EXPERIMENT XXII.b, Pro-OMe.HCl (1.65 g, 10 mmol) and NEt_3 (1.8 ml, 13 mmol) gave 0.918 g (69%) of Pro-OMe as an oil, which was used without delay.

g. L-Glutamic acid - Dimethyl Ester HCl (Glu-(γ -OMe)-OMe)HCl :

Dry HCl was passed through a refluxing solution of L-Glutamic acid (5 g, 34 mmol) in dry methanol (75 ml) for 3 h, concentrated to 20-30 ml, triturated with dry ether (50 ml x 3), filtered and crystallized from dry methanol ether to give 4.4 g (61%) of Glu(γ -OMe).HCl, mp. 114°C. (lit.[59] mp. 116°C).

ir : ν_{max}(KBr) cm^{-1}: 3000 (br, salt), 1750 (ester).

h. Glu-DiOMe :

Under conditions described in EXPERIMENT XXII b, Glu-DiOMe.HCl (0.424 g, 2 mmol) and NEt_3 (0.28 ml, 2 mmol) gave 0.27 g (77%) of Gludi-OMe as thick syrup, which was used without delay.

i. Glutamic Acid-γ-Methyl Ester : Glu(γ-OMe)-OH :

Thionylchloride (7.2 ml, 100 mmol) was added, in drops, to stirred and ice-cooled dry MeOH (120 ml) followed by, in small lots, Glutamic acid (14.7 g, 100 mmol). The reaction mixture was allowed to attain rt, left stirred for 0.5 h, cooled to 5°C, admixed with, in drops, NEt_3 (35 ml, 250 mmol), filtered and crystallized from MeOH-H_2O (80:20) to give 13.0 g (80%), mp. 176°C. (lit.[60] mp. 175°).

ir : ν_{max}(KBr) cm^{-1} : 1745 (ester), 1720 (acid).

ms : m/z : 162 $(M+1)^+$.

XXV. The Preparation of N-Benzoyl Amino Acids :

a. N-Benzoyl-Glycine (BzGly) :

Benzoyl chloride (5.8 ml, 50 mmol) and 4N-NaOH (15 ml) was simultaneously added to an ice-cooled and stirred suspension of Glycine (3.75 g, 50 mmol) in 4N-NaOH (12.5 ml). The addition was controlled to keep the medium basic throughout. After 0.5 h of additional stirring the reaction mixture was extracted with ether, adjusted to pH 3, filtered and crystallized from EtOAc-hexane to give 6.93 g (77%) of BzGly, mp. 187-188°C. (lit.[61] mp. 189°).

ir : ν_{max}(KBr) cm^{-1} : 3400 (NH), 1730 (COOH), 1610, 1510 (amide).

b. N-Benzoyl-Leucine : (BzLeu-OH) :

(i) Cyclohexylamine salt of BzLeu-OH :

Benzoyl chloride (5.8 ml, 50 mmol) and 2N-NaOH (25 ml) was simultaneously added to an ice-cooled and stirred suspension of L-Leucine (6.55 g, 50 mmol) in 2N-NaOH (25 ml). The addition of alkali was controlled to keep medium basic throughout. After 0.5 h of additional stirring, the reaction mixture was extracted with ether, adjusted to pH 3, extracted with ether, dried, admixed with cyclohexylamine (10 ml), evaporated and the residue on crystallization from MeOH-Et_2O gave 9.11 g (54%) of the salt, mp. 156°C.

(ii) BzLeu-OH :

A suspension of the cyclohexylamine salt of Bz-Leu-OH (6.15 g, 18.4 mmol) in EtOAc (50 ml) was admixed with 2N-HCl (50 ml), shaken, layers separated, the aqueous portion extracted with EtOAc, the combined extracts dried, solvents evaporated in vacuo and the residue on crystallization from EtOAc-hexane gave 3.18 g (73%) of BzLeu-OH, mp. 104°C. (lit.[62] mp. 102°C).

ir : ν_{max}(KBr) cm^{-1} : 3290 (-NH), 1720 (acid), 1630, 1520 (amide).

nmr : δ($CDCl_3$) : 1.0 (d, 6H, $CH(C\underline{H}_3)_2$), 1.6 (br, 1H, $C\underline{H}(CH_3)_2$), 1.8 (br, 2H, $CH\text{-}C\underline{H}_2$), 4.8 (br, t, 1H, tert.proton), 6.9 (br, d, 1H, NH), 7.6 (m, 5H, aromatic).

XXVI. The Preparation of N-Benzoyl-α-Amino Acid Methyl Esters :

a.N-Benzoyl Glycine Methyl Ester (BzGly-OMe) :

Benzoyl chloride (0.23 ml, 2.3 mmol) was added, in drops, to an ice-cooled and stirred solution of Gly-OMe.HCl (0.251 g, 2 mmol) in saturated $NaHCO_3$

(25 ml), keeping the medium basic throughout. The reaction mixture was left stirred for 2 h, extracted with ether, adjusted to pH 3, extracted with EtOAc (3 x 25 ml), dried and evaporated to give 0.176 g (91%) of BzGly-OMe, mp.84-5°C.

ir : ν_{max}(KBr) cm^{-1} : 3290 (-NH), 1735 (ester), 1630, 1555 (amide).

nmr : δ ($CDCl_3$) : 3.7 (s, 3H, $COOC\underline{H}_3$), 4.2 (d, 2H, $NHC\underline{H}_2$), 7.0 (br, 1H, NH), 7.6 (m, 5H, aromatic).

b.N-Benzoyl Leucine Methyl Ester (BzLeu-OMe) :

Benzoyl chloride (1.46 g, 10.25 mmol) was added, in drops, to an ice-cooled and stirred solution of Leu-OMe.HCl (1.5 g, 8.25 mmol) in saturated $NaHCO_3$ (~75 ml), keeping the medium basic throughout. The mixture was left stirred vigorously for 3 h, extracted with ether, dried, evaporated and the residue on crystallization from benzene-hexane gave 1.28 g (62%) of BzLeu-OMe, mp.103°C. (lit.[63] mp. 104°C).

ir : ν_{max}(KBr) cm^{-1} : 3320 (NH), 1760 (ester), 1650, 1550 (amide).

c. N-Benzoyl Proline Methyl Ester (BzPro-OMe) :

Benzoyl chloride (4.2 ml, 36 mmol) was added, in drops, to an ice-cooled and stirred solution of Pro-OMe.HCl (4.96 g, 30 mmol) in saturated $NaHCO_3$ (300 ml), keeping the medium basic throughout. The mixture was left stirred for 10 h, extracted with EtOAc (3 x 50 ml), dried, evaporated and the residue on crystallization from benzene-hexane to give 3.87 g (56%) of BzPro-OMe, mp. 89°C. (lit.[58] mp. 89-90°).

ir : ν_{max}(KBr) cm^{-1} : 3450 (NH), 1735 (ester), 1620 (amide).

nmr : δ ($CDCl_3$) : 1.9 - 2.4 (m, 4H, $(C\underline{H}_2)_2$), 2.7 (m, 2H, N-$C\underline{H}_2$), 3.9 (s, 3H, $COOC\underline{H}_3$), 4.7 (m, 1H, tert.proton), 7.5 (br, 5H, aromatic).

d. N-Benzoyl Glutamic Acid Di-Methyl Ester (BzGlu-diOMe) :

Benzoyl chloride (0.12 ml, 1.15 mmol) was added to an ice-cooled and stirred solution of Glu-diOMe.HCl (0.212 g, 1 mmol) in saturated $NaHCO_3$ (15 ml), keeping the medium basic throughout. The mixture was left stirred for 4 h, extracted with EtOAc (3 x 25 ml), dried, evaporated and the residue on crystallization from EtOAc-hexane to give 0.170 g (60%) of BzGlu-diOMe, mp. 79-81°C. (lit.[64] mp.83°).

ir : ν_{max}(KBr) cm^{-1} : 3290 (-NH), 1750 (ester), 1645, 1550 (amide).

nmr : δ ($CDCl_3$) : 2.2 - 2.7 (m, 4H, $C\underline{H}_2$-$C\underline{H}_2$), 3.6 (s, 3H, γ -$COOC\underline{H}_3$), 3.75 (s, 3H, α -$COOC\underline{H}_3$), 4.8 (m, 1H, tert.proton), 7.0 (br, 1H, NH), 7.6 (m, 5H, aromatic).

XXVII. The Preparation of Selectively C-Protected Glutamic Acids :

a.N-Benzoyl Glutamic Acid-α -Methyl Ester (Bz-Glu(α -OMe)-OH :

A solution of Bz-Pro-OMe (2.796 g, 12 mmol) in CCl_4 (40 ml) was admixed with CH_3CN : H_2O (1:3, 160 ml), followed by $NaIO_4$ (46 g, 215 mmol). The mixture was shaken mechanically for 1 h, treated with $RuCl_3.3H_2O$ (0.090 g, 2.2 mol%), left shaken for 24 h, filtered, the residue washed with CCl_4, layers separated, aqueous layer extracted with EtOAc (3 x 100 ml), the organic extracts combined, dried and evaporated. The resulting residue was stirred with saturated $NaHCO_3$ (90 ml) for 2 h, extracted with EtOAc (3 x 100 ml) to recover 1.7 g of the neutral starting material and 0.712 g (61%) of Bz-pyro-glutamate, mp. 154°C . The aqueous layer was adjusted to pH 3, saturated with NaCl, extracted

with EtOAc (3 x 50 ml), dried and evaporated to give 0.309 g (25%) of BzGlu-(α-OMe)-OH, as a thick syrup.

ir : ν_{max}(KBr) cm^{-1} : 3300 (NH), 1740 (ester), 1620, 1520 (amide).

nmr : δ($CDCl_3$) : 1.92 - 2.9 (m, 4H, $C\underline{H}_2$-$C\underline{H}_2$), 3.75 (s, 3H, α -COO$C\underline{H}_3$), 4.8 (br, 1H, tert.proton), 7.3 - 7.9 (m, 5H, aromatic), 9.6 (br, 2H, NH, COOH).

b. N-Benzoyl Glutamic Acid-γ -Methyl Ester (BzGlu(γ -OMe)-OH) :

Benzoyl chloride (2.25 ml, 19.2 mmol) was added, in drops to an ice-cooled and stirred solution of Glu(γ-OMe)-OH (2.64 g, 16.48 mmol) in saturated $NaHCO_3$(~ 175 ml), keeping the medium basic. The reaction mixture was left stirred for 3 h, adjusted to pH 3, cooled, saturated with NaCl, extracted with EtOAc (3 x 50 ml), dried, evaporated and the residue on crystallization from acetone-hexane gave 3.04 g (70%) of BzGlu(γ-OMe)-OH, mp. 104-6C. (lit.[65] mp. 107°C.).

ir : ν_{max}(KBr) cm^{-1} : 3340 (NH), 1740 (ester), 1330 (br, ester+acid), 1650, 1640 (amide).

nmr : δ($CDCl_3$) : 2.1 - 2.7 (br, 4H, $C\underline{H}_2$-$C\underline{H}_2$), 3.6 (s, 3H, COO$C\underline{H}_3$), 4.8 (br, 1H, tert.proton), 7.3 - 7.9 (m, 5H, aromatic), 8.0 (br, 1H, NH), 9.7 (br, 1H, COOH).

XXVIII. The Preparation of Authentic Dipeptides :

a. N-Benzoyl-Leucyl-Leucine-Methyl Ester : Bz-Leu-Leu-OMe (30) :

1-Hydroxybenzotriazole(HOBt)(0.270 g, 2 mmol), followed by a solution of DCC (0.412 g, 2 mmol) in CH_2Cl_2 (5 ml) was added to a stirred solution of BzLeu (0.470 g, 2 mmol) in dry CH_2Cl_2 (10 ml). A solution of Leu-OMe - freshly

prepared by dropwise addition of NEt_3 (0.28 ml, 2 mmol) to an ice-cooled and stirred solution of Leu-OMe.HCl (0.364 g, 2 mmol) in dry CH_2Cl_2 (10 ml) and leaving aside for 0.5 h - was then added. The mixture was left stirred overnight, filtered, washed with CH_2Cl_2, the filtrate and washings evaporated, the residue dissolved in EtOAc, washed with 2N-HCl (2 x 25 ml), satd. $NaHCO_3$ (2 x 25 ml), satd. NaCl, dried, evaporated and chromatographed on silica gel. Elution with benzene : EtOAc (70:30) gave 0.280 g (39%) of (30), mp. 200°C.

anal : Calcd. for $C_{20}H_{30}N_2O_4$ (Mol.wt. 362)
C, 66.29; H, 8.28; N, 7.73%
Found : C, 66.58; H, 8.80; N, 7.70%

ir : ν_{max}(KBr) cm^{-1} : 3320, 3260 (NH), 1740 (ester), 1620, 1520 (amide).

nmr : δ($CDCl_3$) : 0.9 (d, d, 12H, $CH(C\underline{H}_3)_2)_2$, 1.7 (m, 6H, $(C\underline{H}_2\text{-}C\underline{H}Me_2)_2$, 3.75 (s, 3H, $COOC\underline{H}_3$), 4.7 (m, 2H, tert.protons), 6.75 (br, 2H, 2 x NH), 7.6 (m, 5H, aromatic).

ms : m/z : 363(MH^+), 362 (M^+), 306 (M^+-(isobutylene)), 218 (M^+-NH CH(E)-CH_2ipr), 190 $(BzNHCHCH_2ipr)^+$.

b. N-Benzoyl-Leucyl-Glutamic Acid Di-Methyl Ester : BzLeu-Glu(γ-OMe)-OMe (31) :

1-Hydroxybenzotriazole (HOBt) (0.270 g, 2 mmol), followed by DCC (0.412 g, 2 mmol) was added to a stirred solution of BzLeu (0.470 g, 2 mmol) in dry CH_2Cl_2 (10 ml). A solution of Glu-DiOMe - freshly prepared by dropwise addition of NEt_3 (0.28 ml, 2 mmol) to an ice-cooled and stirred solution of Glu-diOMe.HCl (0.424 g, 2 mmol) in dry CH_2Cl_2 (10 ml) and leaving aside for 0.5 h - was then added, the mixture left stirred overnight at rt, filtered, washed with CH_2Cl_2, the filtrate and washings evaporated, the residue dissolved in

EtOAc, washed with 2N-HCl (2 x 25 ml), satd. $NaHCO_3$, satd. NaCl, dried, evaporated and the residue on crystallization from benzene-hexane gave 0.376 g (48%) of (31), mp. 119-120°C.

anal : Calcd. for $C_{20}H_{28}N_2O_6$ (Mol.wt 392)
C, 61.22; H, 7.14; N, 7.14%
Found : C, 61.71; H, 6.80; N, 7.54%

ir : ν_{max}(KBr) cm^{-1} : 3250 (NH), 1735 (ester), 1625, 1525 (amide).

nmr : δ ($CDCl_3$) : 1.0 (d, 6H, CH-$\underline{Me}_2$), 1.6 - 2.5 (m, 7H, methylenes, $\underline{CH}$-Me_2), 3.58, 3.7 (s, s, 3H, α -COO$\underline{Me}$), 3.6 (s, 3H, γ -COO$\underline{CH}_3$), 4-5 (m, 2H, tert.protons), 6.7 - 7.9 (m, 7H, aromatic, 2 x NH).

ms : m/z : 392 (M^+), 336 (M H^+-(isobutylene)), 218 (M^+-NH-CH(E)CH_2CH_2(E)), 190 (BzNHCHCH_2ipr).

c. N-Benzoyl-γ-Methyl-L-Glutamyl-Leucine-Methyl Ester : (BzGlu(γ-OMe)-Leu-OMe (32) :

1-Hydroxybenzotriazole (HOBt) (0.135 g, 1 mmol) followed by a solution of DCC (0.206 g, 1 mmol) in dry CH_2Cl_2 (5 ml) was added to a stirred solution of BzGlu(γ-OMe)-OH (0.265 g, 1 mmol) in dry CH_2Cl_2 (5 ml). A solution of Leu-OMe - freshly prepared by dropwise addition of NEt_3 (0.14 ml, 1 mmol) to an ice-cooled and stirred solution of Leu-OMe.HCl (0.182 g, 1 mmol) in dry CH_2Cl_2 (5 ml) and leaving aside for 0.25 h - was then added. The mixture was left stirred for 24 h, filtered, washed with EtOAc, evaporated, the residue dissolved in EtOAc, washed with 2N.HCl (25 ml), satd. $NaHCO_3$ (25 ml), water (20 ml), dried and evaporated to give 0.177 g (45%) of (32), as a thick syrup.

anal : Calcd. for $C_{20}H_{28}N_2O_6$(Mol.wt. 392).

C, 61.2; H, 7.11; N, 7.11%

Found : C, 61.05; H, 7.65; N, 7.47%

ir : ν_{max}(neat) cm^{-1} : 3280 (NH), 1730 (ester), 1520, 1640 (amide).

nmr : $\delta(CDCl_3)$: 0.9 (d, 6H, $CH\underline{Me}_2$), 1.4 (m, 1H, $C\underline{H}$-$(CH_3)_2$), 1.6 (m, 2H, $C\underline{H}_2$-$CHMe_2$), 2.2 - 2.9 (m, 4H, CH-$C\underline{H}_2$-$C\underline{H}_2$COOMe), 3.65 (s, 3H, -$COOC\underline{H}_3$), 3.75 (s, 3H, α-$COOC\underline{H}_3$), 4.7 (m, 2H, tert.protons), 7.1 - 7.8 (m, 7H, 2 x NH + aromatic).

ms : m/z : 393(MH^+), 248 (M^+-NHCH(E)CH_2ipr), 220 $(BzNHCHCH_2CH_2E)^+$.

d. N-Benzoyl- γ -Methyl-Glutamyl-Glutamic Acid-Di-Methyl Ester : BzGlu(γ-OMe)-Glu(γ -OMe)-OMe (33) :

1-Hydroxybenzotriazole (HOBt) (0.135 g, 1 mmol) followed by a solution of DCC (0.206 g, 1 mmol) in dry CH_2Cl_2 (5 ml) was added to a stirred solution of Bz-Glu(γ -OMe)-OH (0.265 g, 1 mmol) in dry CH_2Cl_2 (5 ml). A solution of Glu(γ-OMe)-OMe - freshly prepared by dropwise addition of NEt_3 (0.14 ml, 1 mmol) to an ice-cooled and stirred solution of Glu-diOMe.HCl (0.212 g, 1 mmol) in dry CH_2Cl_2 (5 ml) and leaving aside for 0.5 h - was then added. The mixture was left stirred overnight, filtered, washed with CH_2Cl_2, evaporated, dissolved in EtOAc, washed with 2N-HCl (20 ml), satd. $NaHCO_3$(20 ml), water (20 ml), dried, evaporated and the residue on crystallization from EtOAc-hexane gave 0.290 g (68%) of (33), mp. 119-120°C.

anal : Calcd. for $C_{20}H_{26}N_2O_8$ (Mol.wt.422).

C, 56.87; H, 6.16; N, 6.63%

Found : C, 56.30; H, 6.66; N, 7.10%

ir : ν_{max}(KBr) cm^{-1} : 3280, 3215 (-NH), 1740, 1730 (ester), 1620, 1520 (amide).

nmr : δ ($CDCl_3$) : 2.0-2.7 (m, 8H, methylenes), 3.6, 3.65 (s, s, 3H, 3H, 2 x γ-$COOCH_3$), 3.7 (s, 3H, α-$COOCH_3$), 4.7 (m, 2H, tert.protons), 7.3-7.9 (m, 5H, aromatic).

ms : m/z : 423 MH^+, 220 $(BzNHCHCH_2CH_2COOMe)^+$, 174 (NHCH(E)-CH_2CH_2(E)).

e. N-BenzoylGlutamyl(α-OMe)-Glutamic Acid Di-methyl Ester : BzGlu(α-OMe)-Glu(γ-OMe)-OMe (34) :

1-Hydroxybenzotriazole (HOBt) (0.034 g, 0.25 mmol), followed by a solution of DCC (0.050 g, 0.25 mmol) in dry CH_2Cl_2 (2 ml) was added to a stirred solution of BzGlu(α-OMe)-OH (0.066 g, 0.25 mmol) in dry CH_2Cl_2 (2 ml). A solution of Glu(γ-OMe)-OMe - freshly prepared by dropwise addition of NEt_3 (0.050 ml, 0.375 mmol) to an ice-cooled and stirred solution of Glu(γ-OMe)-OMe. HCl (0.053 g, 0.25 mmol) in dry CH_2Cl_2 (5 ml) and leaving aside for 0.25 h - was then added. The mixture was left stirred overnight, filtered, washed with CH_2Cl_2, the combined filtrates evaporated, the residue dissolved in EtOAc, washed with 2N-HCl (10 ml), 1M-Na_2CO_3 (10 ml), water (10 ml), dried and evaporated to give 0.048 g (45%) of (34), as a sticky solid.

ir : ν_{max}(neat) cm^{-1} : 3290 (-NH), 1750 (ester), 1550, 1645 (amide).

nmr : δ ($CDCl_3$) : 1.9-2.7 (m, 8H, methylenes), 3.6 (s, 3H, γ-$COOCH_3$), 3.62 (s, 3H, α-$COOCH_3$), 3.7 (s, 3H, α-$COOCH_3$), 4.7 (m, 2H, tert.protons), 7.32-7.9 (m, 7H, aromatic + 2 x NH).

ms : m/z : 363 (M^+-COOMe), 220 $(CH_2CH_2CHCOC_6H_5NHCO_2Me)^+$, 174 $(NH\text{-}CHCO_2Me\text{-}CH_2CH_2CO_2Me)^+$.

f. N-Benzoyl-γ-Methyl-L-Glutamyl-Glycine-Methyl Ester : BzGlu(γ-OMe)-Gly-OMe (35) :

1-Hydroxybenzotriazole (HOBt) (0.135 g, 1 mmol), followed by a solution of DCC (0.2069, 1 mmol) in CH_2Cl_2 (5 ml) was added to a stirred solution of BzGlu (γ-OMe-OH) (0.265 g, 1 mmol) in dry CH_2Cl_2 (5 ml). A solution of Gly-OMe - freshly prepared by dropwise addition of NEt_3 (0.14 ml, 1 mmol) to an ice-cooled and stirred solution of Gly-OMe.HCl (0.125 g, 1 mmol) in dry CH_2Cl_2 (5 ml) and leaving aside for 0.5 h - was then added. The mixture was left stirred for 24 h, filtered, washed with CH_2Cl_2, evaporated, the residue dissolved in EtOAc, washed with 2N.HCl (25 ml), satd. $NaHCO_3$ (25 ml), satd. NaCl, dried and evaporated to give 0.114 g (34%) of (35), as a thick syrup.

anal : Calcd. for $C_{16}H_{20}N_2O_6$ (Mol.wt. 336).
C, 57.14; H, 5.95; N, 8.33%
Found : C, 57.62; H, 6.02; N, 7.88%

ir : ν_{max}(neat) cm^{-1} : 3340, 3280 (-NH), 1750 (ester), 1650, 1540 (amide).

nmr : δ ($CDCl_3$) : 2.1-2.8 (m, 4H, methylenes), 3.6 (s, 3H, γ-$COOCH_3$), 3.7 (s, 3H, α-$COOCH_3$), 4.0 (d, 2H, NH-CH_2-COOMe), 4.8 (m, 1H, tert.proton), 7.2-7.9 (m, 7H, aromatic + 2 x NH).

ms : m/z : 248 (M^+-$NHCH_2COOMe$), 220 ($BzNHCHCH_2CH_2CO_2Me$).

g. N-Benzoyl-α-Methyl-L-Glutamyl-Glycine Methyl Ester : BzGlu(α-OMe)-Gly-OMe (36) :

1-Hydroxybenzotriazole (HOBt) (0.0675 g, 0.5 mmol), followed by a solution of DCC (0.103 g, 0.5 mmol) in dry CH_2Cl_2 (2 ml) was added to a stirred solution of BzGlu(α-OMe)-OH (0.1325 g, 0.5 mmol) in dry CH_2Cl_2 (5 ml).

A solution of Gly-OMe - freshly prepared by dropwise addition of NEt_3 (0.1 ml), 0.75 mmol) to an ice-cooled and stirred solution of Gly-OMe.HCl (0.063 g, 0.5 mmol) in dry CH_2Cl_2 (5 ml) and leaving aside for 0.5 h - was then added. The mixture was left stirred overnight, filtered, washed with CH_2Cl_2, the filtrate and washings evaporated, the residue dissolved in EtOAc, washed with 2N-HCl (10 ml), satd. $NaHCO_3$ (10 ml), water (10 ml), dried and evaporated to give 0.086 g (52%) of (36), mp. 101°C.

ir : ν_{max}(KBr) cm^{-1} : 3320 (-NH), 1740 (ester), 1640, 1540 (amide).

nmr : δ($CDCl_3$) : 1.9-2.5 (m,4H, methylenes), 3.65 (s, 3H,α-COOMe), 3.7 (d, 2H, $NHCH_2$-COOMe), 3.8 (s, 3H,α-$COOCH_3$), 4.6 (m, 1H, tert.proton), 7.3-7.8 (m, 5H, aromatic).

ms : m/z : 143 $(M\ H^+ - BzNHCH_2COOMe)^+$.

h. N-BenzoylGlycyl-Glycine-Methyl Ester (BzGly-Gly-OMe) (37) :

1-Hydroxybenzotriazole (HOBt) (0.270 g, 2 mmol), follwed by a solution of DCC (0.412 g, 2 mmol) in dry CH_2Cl_2 (5 ml) was added to a stirred solution of BzGly (0.358 g, 2 mmol) in dry CH_2Cl_2: DMF (10:1, 11 ml). A solution of Gly-OMe - freshly prepared by dropwsie addition of NEt_3 (0.28 ml, 2 mmol) to an ice-cooled and stirred solution of Gly-OMe.HCl (0.251 g, 2 mmol) and leaving aside for 0.5 h - was then added. The mixture was left stirred overnight, filtered, washed with CH_2Cl_2 - the combined filtrates evaporated, dissolved in EtOAc, washed with 2N-HCl (2 x 25 ml), satd. $NaHCO_3$ (2 x 25 ml), satd. NaCl solution, dried, evaporated and residue on crystallization from benzene gave 0.280 g (56%) of (37), mp. 111-112°C.

anal : Calcd. for $C_{12}H_{14}N_2O_4$ (Mol.wt. 250)

C, 57.6, H, 5.6, N, 11.2%

Found : C, 57.82; H, 5.90; N, 11.61%

ir : ν_{max}(KBr) cm^{-1} : 3290 (NH), 1730 (ester), 1540, 1640 (amide).

nmr : $\delta(CDCl_3)$: 3.7 (s, 3H, $CO_2\underline{CH_3}$), 4.2 (d, d, 4H, $\underline{CH_2}$-$\underline{CH_2}$), 7.3 -7.9 (m, 5H, aromatic).

ms : m/z : 162 $(BzNHCH_2CO)^+$, 134 $(BzNHCH_2)^+$.

i. N-Benzoyl-Glycyl-Glutamic Acid DiMethyl Ester : BzGly-Glu(γ-OMe)-OMe (38) :

1-Hydroxybenzotriazole (HOBt) (0.270 g, 2 mmol) followed by a solution of DCC (0.412 g, 2 mmol) in dry CH_2Cl_2 (5 ml) was added to a stirred solution of BzGly (0.358 g, 2 mmol) in dry CH_2Cl_2 : DMF (10:1, 11 ml). A solution of Glu(γ-OMe)-OMe - freshly prepared by dropwsie addition of NEt_3 (0.28 ml, 2 mmol) to an ice-cooled and stirred solution of Glu(γ-OMe)-OMe.HCl (0.424 g, 2 mmol) in CH_2Cl_2 (10 ml) and leaving aside for 0.25 h - was then added. The mixture was left stirred for 12 h at rt, filtered, washed with EtOAc, the combined filtrates evaporated, dissolved in EtOAc, filtered, washed with 2NHCl (2 x 25 ml), satd. $NaHCO_3$ (2 x 25 ml), water, dried, evaporated and the residue on crystallization from benzene gave 0.421 g (62%) of (38), mp. 107°C.

ir : ν_{max}(KBr) cm^{-1} : 3280 (NH), 1740 (ester), 1660, 1630, 1540 (amide).

nmr : $\delta(CDCl_3)$: 1.9-2.6 (m, 4H, -CH$\underline{CH_2}$-$\underline{CH_2}$-$COOCH_3$), 3.6 (s, 3H, γ-$COO\underline{CH_3}$), 3.7 (s, 3H, α-$COO\underline{CH_3}$), 4.2 (d, 2H, NH-$\underline{CH_2}$), 4.6 (m, 1H, tert.proton), 7.2 -7.9 (m, 7H, aromatic + 2 NH).

XXIX. The Reaction of Free Amino Acids With Equivalent Amounts of the Water soluble carbodiimide (22) :

a. Reaction of L-Leucine with (22) :

A solution of L-Leucine (0.262 g, 2 mmol) in H_2O (22 ml) was admixed with (22) (0.790 g, 2 mmol), the clear solution left stirred for 2d, cooled adjusted to pH >10, benzoylated with BzCl (0.338 g, 2.4 mmol), left stirred for 5h, acidified with 2N.HCl to pH 3, extracted with EtOAc, dried, evaporated, the residue dissolved in MeOH, treated with ethereal CH_2N_2, evaporated and the residue chromatographed on silica gel. Elution with benzene : EtOAc (70:30) gave 0.104 g (21%) of BzLeuOMe and 0.070 g (20%) of BzLeu-Leu-OMe, mp. respectively 94°C and 188°C.

b. Reaction of Glutamic Acid with (22) :

A stirred solution of L-Glutamic acid (0.147 g, 1 mmol) in water (10 ml) was admixed with (22) (0.395 g, 1 mmol). The clear solution was left stirred for 2 d, adjusted to pH >10, admixed with BzCl (0.169 g, 1.2 mmol), left stirred for 5 h, cooled, acidified with 2N.HCl to pH ~ 3, extracted with EtOAc, dried, evaporated, dissolved in MeOH, treated with ethereal CH_2N_2 and evaporated, HPLC of the residue (0.102 g) showed as relative percentages BzGlu(α -OMe)-GludiOMe (54.6%), BzGlu(γ -OMe)-GludiOMe (44.6%) and negligible amounts of BzGludiOMe (0.5%).

c. Reaction of Glycine with (22) :

A stirred solution of Glycine (0.075 g, 1 mmol) in H_2O (9 ml) was admixed with (22) (0.395 g, 1 mmol). The clear solution was left stirred for 2 d, adjusted to pH >10, admixed with BzCl (0.169 g, 1.2 mmol), left stirred for 5 h, cooled, acidified with 2N-HCl to pH 3, filtered, dried, dissolved in MeOH, treated with ethereal CH_2N_2 and evaporated, HPLC of the residue (0.062 g),

showed that the product largely consisted of BzGly-Gly-OMe.

d. Reaction of L-Lysine with (22) :

Compound (22) (0.790 g, 2 mmol) was added to an ice-cooled and stirred solution of L-Lysine.HCl (0.364 g, 2 mmol) in 2N-NaOH (2 ml, 4 mmol). The reaction was made clear by the addition of water (10 ml), the clear solution left stirred for 2 d, adjusted to pH >10, admixed with BzCl (0.338 g, 2.4 mmol), left stirred for 5 h, acidified with 2N.HCl to pH 3, filtered, dried, dissolved in MeOH, treated with ethereal CH_2N_2 evaporated to give 0.202 g of residue. HPLC showed the presence of a major product and NMR strongly supported for the product, the structural assignments, Bz-Lys(ω Bz)-Lys(ω Bz)-OMe.

nmr : $\delta(CDCl_3)$: 1-2 (m, 12H, methylenes), 3.25-3.6 (m, 4H,$(CH_2\text{-}\underline{CH_2}\text{-}NH\text{-}Bz)_2$, 3.75 (s, 3H, $COO\underline{CH_3}$), 4.75 (m, 2H, tert.protons),. 7.25-7.8 (m, 15H, aromatic).

XXX. The Reaction of Glu And Leu In Water In The Presence Of Water Soluble Carbodiimide (22) :

a. Isolation of Product by Chromatography :

A solution of L-Leucine (0.262 g, 2 mmol) L-Glutamic acid (0.294 g, 2 mmol) in H_2O (25 ml) was admixed with (22) (0.790 g, 2 mmol), the clear solution left stirred for 2 d, cooled adjusted to pH >10, benzoylated with BzCl (0.338 g, 2.4 mmol), left stirred for 5 h, acidified with 2N-HCl to pH 3, extracted with EtOAc, dried, evaporated, the residue dissolved in MeOH, cooled, treated with ethereal CH_2N_2, evaporated and the residue chromatographed on silica gel. Elution with PhH : EtOAc (70:30) followed by preparative tlc gave 0.250 g (32%) of BzGlu(γ-OMe)-Leu-OMe (32), mp. 109°C. This compound was identical

in all respects to an authentic sample.

b. Analysis of Product by HPLC :

The residue obtained from (Experiment XXX a) on HPLC analysis gave the following percentage composition :

BzGlu(γ-OMe)-Leu-OMe	72%
BzGlu(α-OMe)-Gludi-OMe	15%
BzLeu-Leu-OMe	8%
Higher peptides	1.5%

XXXI. The Reaction of Glycine And Glutamic Acid In Water In The Presence of Water Soluble Carbodiimide (22) :

A solution of Glycine (0.075 g, 1 mmol), L-Glutamic acid (0.147 g, 1 mmol) in water (16 ml) was admixed with (22) (0.395 g, 1 mmol), the clear solution left stirred for 2 d, cooled, adjusted to pH >10, benzoylated with BzCl (0.169 g, 1.2 mmol) left stirred for 5 h, acidified with 2N-HCl to pH 3, filtered, dried, dissolved in MeOH, cooled, treated with ethereal CH_2N_2, evaporated. HPLC of the residue (0.130 g) showed that the product largely consisted of BzGly-Gly-OMe and higher peptides.

XXXII. The Reaction Of Glutamic Acid And L-Lysine.HCl In Water In The Presence Of Water Soluble Carbodiimide (22) :

Compound (22) (0.790 g, 2 mmol) was added to an ice-cooled and stirred solution of L-Lysine.HCl (0.364 g, 2 mmol) in 2N-NaOH (2 ml), followed by the addition of L-Glutamic acid (0.294 g, 2 mmol) in water (16 ml). The

clear solution left stirred for 2 d, adjusted to pH >10, admixed with BzCl (0.338 g, 2.4 mmol), left stirred for 5 h, cooled, acidified with 2N-HCl to pH 3, extracted with EtOAc, dried, evaporated, dissolved in MeOH, cooled, treated with ethereal CH_2N_2, evaporated. HPLC of the residue 0.178 g, showed presence of three products in the ratio 0.38 : 13.2 : 80. The nmr suggests for the major product the structure BzNH-Lys(ω Bz)-Gludi-OMe.

XXXIII. Preparation Of Dioctadecylcarbodiimide (DODCI) (39) :

a. Dioctadecylthiourea (40) :

A stirred solution of CS_2 (0.3 ml, 5 mmol) and octadecylamine (2.69 g, 10 mmol) in dry EtOH (40 ml) was refluxed for 12 h, cooled, filtered and crystallized from EtOH to give 2.45 g (86%) of (40), mp. 96-97°C.

anal : Calcd. for $C_{37}H_{76}N_2S$ (Mol.wt. 580).
C, 76.55; H, 13.10; N, 4.82%
Found : C, 76.86; H, 13.33; N, 5.02%

ir : ν_{max}(KBr) cm^{-1} : 3280 (-NH), 1590, 1490, 1375 (thiourea).

ms : m/z : 580 (M^+-S).

b. Dioctadecylcarbodiimide (39) :

Yellow HgO (1.3 g, 6 mmol) was added to a vigorously stirred mixture of (40) (1.16 g, 2 mmol) in dry CS_2 (8 ml). The reaction mixture was left stirred for 4 h, filtered, washed with dry petroleum ether and evaporated to give 1.08 g (94%) of (39), mp. 49-50°C.

anal : Calcd. for $C_{37}H_{74}N_2$ (Mol.wt. 546).

C, 81.31; H, 13.55; N, 5.13%

Found : C, 81.52; H, 13.81; N, 5.19%

ir : ν_{max}(KBr) cm^{-1} : 2130 (carbodiimide).

nmr : $\delta(CDCl_3)$: 0.7-2.5 (m, 70 H), 3.3, 3.7 (t, t, 4H, N-$\underline{CH_2}$-CH_2).

ms : m/z : 546 (M^+).

XXXIV. Preparation of Amino Acid Esters :

a. L-Aspartic Acid Di-Methyl Ester. HCl (Asp(β -OMe)-OMe.HCl) :

Dry HCl was passed through a refluxing solution of L-Aspartic acid (5 g, 37.5 mmol) in dry methanol (50 ml) for 2 h, concentrated to 20-30 ml, triturated with dry ether (100 ml), filtered, crystallized, from dry methanol and ether to give 6.4 g (65%) of Asp(β -OMe)-OMe.HCl, mp. 114-115°C. (lit.[66] mp. 116-7°C).

ir : ν_{max}(KBr) cm^{-1} : 3400 (salt), 1745 (ester).

b. Asp(β -OMe)-OMe :

Under conditions described in EXPERIMENT XXII b, Asp(β -OMe)-OMe. HCl (0.985 g, 5 mmol) and NEt_3 (0.9 ml, 7 mmol) gave 0.563 g (70%) of Asp-(β -OMe)-OMe as an oil, which was used without delay.

c. L-Tryptophan Methyl Ester. HCl (TrpOMe.HCl) :

To stirred and ice-cooled dry MeOH (25 ml) was added, in drops, $SOCl_2$ (1.9 ml, 26 mmol) followed by L-Trp (2.55 g, 12.5 mmol). The reaction ture was left stirred for 4 h at -5 to 0°C, then at rt overnight, concentrated

to 5 ml, admixed with dry ether until turbid, cooled, filtered and dried to give 2.14 g (68%) of TrpOMe.HCl. mp. 213°C. (lit.[67] mp. 213-214°C).

ir : ν_{max}(KBr) cm^{-1} : 3270 (br, salt), 1740 (ester).

d. L-Tryptophan Methyl Ester (Trp-OMe) :

Under conditions described in EXPERIMENT XXII b, Trp-OMe.HCl (2.5 g, 10 mmol) and NEt_3 (1.8 ml, 13 mmol) gave 1.65 g (75%) of Trp-OMe as an oil, which was used without delay.

XXXV. The Preparation of N-Protected Amino Acids :

a. N-Benzoyl Phenylalanine (BzPhe) :

Benzoyl chloride (0.8 ml, 7 mmol) was added, in drops, to an ice-cooled and stirred solution of L-Phe (0.82 g, 5 mmol) in saturated $NaHCO_3$ (60 ml), keeping the medium basic throughout. The mixture was left stirred for 3 h, cooled adjusted to pH 3, filtered, dried and crystallized from EtOAc to give 1.1 g (82%) of BzPhe, mp. 149°C. (lit.[68] mp.146°)

ir : ν_{max}(KBr) cm^{-1} : 3500 (br, acid), 1550, 1640 (amide).

b. N-Benzoyl Methionine (BzMet) :

Benzoyl chloride (4.65 ml, 40 mmol) was added, in drops, to an ice-cooled and stirred solution of L-Met (5 g, 33.5 mmol) in saturated $NaHCO_3$ (300 ml), keeping the medium basic throughout. The mixture was left stirred vigorously for 3 h, cooled, adjusted to pH 3, filtered, dried, and the residue was crystallized from hot water to give 4.8 g (56%) of BzMet, mp.154°C.

ir : ν_{max}(KBr) cm^{-1} : 3300 (-NH), 1720 (acid), 1630, 1540 (amide).

XXXVI. N-Benzoyl Aspartic Acid-β-Methyl Ester : Bz-Asp(β-OMe)-OH :

Benzoyl chloride (2.25 ml, 19.5 mmol) was added, in drops to an ice-cooled and stirred solution of Asp(β-OMe)-OH.HCl (2.99 g, 16.3 mmol) in saturated $NaHCO_3$ (175 ml), keeping the medium basic. The reaction mixture was left stirred for 3 h, adjusted to pH 3 with 2N-HCl, cooled, saturated with NaCl, extracted with ether (3 x 50 ml), dried, evaporated and the residue on crystallization from benzene gave 2.96 g (72%) of BzAsp(β-OMe)-OH, mp. 125°C. (lit.[69] mp. 125-6°C).

ir : ν_{max}(KBr) cm^{-1} : 3300 (-NH), 1750 (ester), 1720 (acid), 1630, 1520 (amide).

nmr : δ($CDCl_3$) : 3.1 (m, 2H, CH-C<u>H</u>$_2$-COOCH$_3$), 3.7 (s, 3H, COOC<u>H</u>$_3$), 5.1 (m, 1H, tert.proton), 7.3-7.9 (m, 6H, aromatic + NH), 8.5 (s, 1H, COOH).

XXXVII. Peptide Bond Formation With Dioctadecylcarbodiimide (DODCI) : Preparation of N-Benzoyl Phenylalanyl Cyclohexylamide (BzPheCONHC$_6$H$_{11}$) (41) :

DODCI (0.546 g, 1 mmol) was added to a stirred solution of BzPhe (0.269 g, 1 mmol) in dry dioxane (10 ml). A solution of cyclohexylamine (0.11 ml, 1 mmol) in dry dioxane (1 ml) was then added, the mixture left stirred overnight, filtered, washed with dioxane, evaporated and the residue chromatographed on silica gel. Elution with benzene gave unreacted DODCI 0.04 g and with PhH : EtOAc (70 : 30) 0.195 g (55%) of (41), mp. 197-198°C.
This compound was identical in all respects to an authentic sample.

anal : Calcd. for $C_{22}H_{26}N_2O_2$ (Mol.wt. 350).

C, 72.62; H, 7.42; N, 8.00

Found : C, 72.33; H, 7.65; N, 7.82%

ir : ν_{max}(KBr) cm^{-1} : 3310 (-NH), 1650, 1540 (amide).

nmr : δ ($CDCl_3$) : 1.0-2.2 (m, 10 H, cyclohexyl), 3.2 (m, 3H, CH-C<u>H</u>$_2$-Ph + CONH-C<u>H</u>), 4.8 (m, 1H, tert.proton), 7.2 (s, 5H, pH), 7 - 7.9 (m, 5 H, aromatic).

ms : m/z : 350 (M^+), 229 (M^+-BzNH), 224 (M^+-CONH cyclohexyl).

XXXVIII. <u>Peptide Bond Formation With DODCI : Preparation of N-Benzoyl Leucyl--cyclohexylamide (BzLeuCONHC$_6$H$_{11}$) (42)</u> :

DODCI (0.546 g, 1 mmol) was added to a stirred solution of BzLeu (0.235 g, 1 mmol) in dry dioxane (10 ml). A solution of cyclohexylamine (0.11 ml, 1 mmol) in dioxane (2 ml) was then added. The reaction mixture was left stirred for 12 h, filtered, washed with dioxane, evaporated and the residue crystallized from EtOAc-hexane to give 0.230 g (73%) of (42), mp. 179-181°C (lit.[70] mp. 180--181°C). This compound was identical in all respects to an authentic sample.

anal : Calcd. for $C_{19}H_{28}N_2O_2$ (Mol.wt. 316).
C, 72.15; H, 8.86; N, 8.86%
Found : C, 72.60; H, 9.51; N, 9.15%.

ir : ν_{max}(KBr) cm^{-1} : 3300 (-NH), 1620, 1540 (amide).

nmr : δ ($CDCl_3$) : 0.9 (d, 6H, CH(C<u>H</u>$_3$)$_2$), 1-2.2 (m, 13 H, cyclohexyl + C<u>H</u>$_2$-C<u>H</u>(CH$_3$)$_2$), 4.5 (m, 1H, tert.proton), 7.2-7.9 (m, 5H, aromatic).

ms : m/z : 316 (M^+), 260(M^+-isobutylene), 190 (M^+-CONHC$_6$H$_{11}$).

XXXIX. Peptide Bond Formation With DODCI : Preparation of N-Benzoyl-Leucyl-Glycine Methyl Ester (BzLeu-Gly-OMe) (43) :

DODCI (0.546 g, 1 mmol) was added to a stirred solution of BzLeu (0.235 g, 1 mmol) in dry dioxane (10 ml). A solution of Gly-OMe (0.089 g, 1 mmol) in dry dioxane (2 ml) - prepared as described in EXPERIMENT XXII b - was added, the mixture left stirred overnight, filtered, washed with dioxane, evaporated and the residue on crystallization from EtOAc-hexane gave 0.195 g (64%) of (43), mp. 178°C. This compound was identical in all respects to an authentic sample.

ir : ν_{max}(KBr) cm^{-1} : 3300 (-NH), 1745 (ester), 1640, 1450 (amide).

nmr : δ ($CDCl_3$) : 1.0 (d, 6H, $CH(C\underline{H}_3)_2$), 1.4 (m, 1H, $C\underline{H}(CH_3)_2$), 1.7 (m, 2 H, CH -$C\underline{H}_2$-$CHMe_2$), 3.7 (s, 3H, $COOC\underline{H}_3$), 4.1 (d, 2H, NH-$C\underline{H}_2$-CO_2Me), 4.8 (m, 1H, tert.proton), 7-7.9 (m, 7H, aromatic + 2 x NH).

ms : m/z : 307 (M^+), 250 (M^+-CH_2CHMe_2).

XL. Peptide Bond Formation With DODCI : Preparation of N-Benzoyl Methionine Glycine Methyl Ester (BzMet-Gly-OMe (44) :

DODCI (0.546 g, 1 mmol) was added to a stirred solution of BzMet (0.253 g, 1 mmol) in dry dioxane (10 ml). A solution of Gly-OMe (0.089 g, 1 mmol) in dioxane (1 ml) - prepared as described in EXPERIMENT XXII b - was added, the mixture left stirred overnight, filtered, washed with dioxane, evaporated and the residue chromatographed on silica gel. Elution with benzene gave unchanged DODCI 0.05 g and with PhH : EtOAc (70:30) 0.136 g (40%) of (44),

mp. 134-5°C.

This compound was identical in all respects to an authentic sample.

anal : Calcd. for $C_{15}H_{20}N_2O_4S$ (Mol.wt.324).
C, 55.55; H, 6.17; N, 8.64%
Found : C, 55.86; H, 6.42; N, 8.12%

ir : ν_{max}(KBr) cm^{-1} : 3290 (-NH), 1750 (ester), 1560, 1445 (amide).

nmr : δ ($CDCl_3$) : 2.2 (s, 3H, S-C<u>H</u>$_3$), 2.1-2.5 (m, 2H, CH-C<u>H</u>$_2$-CH_2-S-CH_3), 2.8 (t, 2H, CH-CH_2-C<u>H</u>$_2$-S-CH_3), 3.2 (t, 1H, N<u>H</u>, -CH_2), 3.75 (s, 3H, COOC<u>H</u>$_3$), 4.1 (br, 2H, NHCHC<u>H</u>$_2$-), 4.2 (br, 1H, PhCON<u>H</u>-), 5.0 (m, 1H, tert.proton), 7.2-8.1 (m, 7H, aromatic + NH).

ms : m/z : 325 (M^+), 250 (M H^+-CH_2CH_2-S-CH_3).

XLI. <u>The Demonstration Of Peptide Bond Formation At The Micellar Interface: The Preparation of BzLeu-Leu-OMe (30) in Isooctane : Water : AOT : DODCI System</u> :

A solution of AOT (0.44 g, 1 mmol) in Isooctane (10 ml) was admixed with water (0.2 ml) and to the resulting clear solution was added, BzLeu (0.118 g, 0.5 mmol) followed by, under stirring, DODCI (0.273 g, 0.5 mmol). After 0.25 h this solution was added in one lot to stirred Leu-OMe (0.073 g, 0.5 mmol) - prepared as described in EXPERIMENT XXIII b. The reaction mixture was left stirred for 2 d, cooled, adjusted to pH 3 with 2N-H_2SO_4, admixed with EtOAc (50 ml), washed with cold water (2 x 50 ml), saturated $NaHCO_3$ (2 x 25 ml), saturated NaCl (1 x 25 ml), dried, evaporated and the residue subjected to preparative tlc using PhH-EtOAc (70:30) as the developer to give 0.067 g (19%) of (<u>30</u>), mp. 198-199°C. identical in all respects to an authentic sample (EXPERIMENT XXVIIIa.).

XLII. The Demonstration of Peptide Bond Formation At the Micellar Interface : The Preparation Of BzPhe-Phe-OMe (46) in Isooctane : Water : AOT : DODCI system :

A solution of AOT (0.44 g, 1 mmol) in Isooctane (10 ml) was admixed with H_2O (0.2 ml) and to the resulting clear solution was added, BzPhe (0.134 g, 0.5 mmol) followed by, under stirring, DODCI (0.273 g, 0.5 mmol). After 0.25 h, this solution was added in one lot to stirred Phe-OMe (0.089 g, 0.5 mmol) - prepared as described in EXPERIMENT XXIVd. The reaction mixture was left stirred for 2d, and worked up as described in EXPERIMENT XLI. The residue was subjected to preparative tlc using PhH : EtOAc (70:30) as developer to give 0.054 g DODCI (39) and 0.045 g, (26%) of (46), mp. 178-179°C. (lit.[70] mp.180°), identical in all respects to an authentic sample.

ir : ν_{max}(KBr) cm^{-1} : 3320 (-NH), 1745 (ester), 1665, 1640, 1550, 1530 (amide).

XLIII. The Demonstration Of Peptide Bond Formation At The Micellar Interface : The Preparation Of BzPhe-Leu-OMe (48) in Isooctane : H_2O : AOT : DODCI system :

A solution of AOT (0.444 g, 1 mmol) in Isooctane (10 ml) was admixed with H_2O (0.2 ml) and to the resulting clear solution was added, BzPhe (0.134 g, 0.5 mmol) followed by, under stirring, DODCI (0.273 g, 0.5 mmol). After 0.25 h, this solution was added in one lot to stirred Leu-OMe (0.0725 g, 0.5 mmol) - prepared as described in EXPERIMENT XXIIIb. The reaction mixture was left stirred for 2 d and worked up as described in EXPERIMENT XLI. The residue was subjected to preparative tlc using PhH : EtOAc (70 : 30) as developer to give 0.045 g (31%) of (48), mp. 143°C., identical in all respects to an authentic

sample.

ir : ν_{max}(KBr) cm^{-1} : 3310 (-NH), 1760 (ester), 1640, 1550 (amide).

nmr : $\delta(CDCl_3)$: 0.8 (d, 6H, $CH(C\underline{H}_3)_2$), 1.1 (m, 1H, $C\underline{H}(CH_3)_2$), 1.5 (m, 2H, $C\underline{H}_2CH(CH_3)_2$), 3.1 (d, 2H, CH-$C\underline{H}_2$-Ph), 3.6, 3.7 (s, s, 3H, $COOC\underline{H}_3$), 4.5 (br, 1H, tert.proton), 4.9 (q, 1H, tert.proton), 7.2 (s, 5H, -CH_2-$\underline{Ph}$), 6.7-7.7 (m, 7H, 2 x NH, amide $\underline{Ph}$).

XLIV. The Demonstration Of Peptide Bond Formation At The Micellar Interface : The Preparation Of BzTrp-Trp-OMe (49) in Isooctane : H_2O: AOT : DODCl system :

A solution of AOT (0.44 g, 1 mmol) in Isooctane (10 ml) was admixed with water (0.2 ml) and to the clear solution was added, BzTrp (0.154 g, 0.5 mmol) followed by, under stirring, DODCl (0.273 g, 0.5 mmol). After 0.25 h this solution was added in one lot to stirred Trp-OMe (0.109 g, 0.5 mmol) - prepared as described in EXPERIMENT XXXIVd. The reaction mixture was left stirred for 2 d and worked up as described in EXPERIMENT XLI. The residue was chromatographed on silica gel. Elution with PhH and PhH : EtOAc (70:30) gave respectively, 0.073 g of DODCl (39) and 0.074 g (40%) of (49), mp. 188-190°C., identical in all respects to an authentic sample.

ir : ν_{max}(KBr) cm^{-1} : 3400, 3320 (-NH), 1740 (ester), 1640, 1610, 1520 (amide).

nmr : $\delta(CDCl_3)$: 2.9 (m, 4H, 2 x $CHC\underline{H}_2$-indole), 3.5 (s, 3H, $COOC\underline{H}_3$), 4.6 (m, 2H, tert.protons), 6.1-8.0 (m, 19 H, 2 x indole + 2 x NH + Ph).

XLV. The Demonstration Of Peptide Bond Formation At The Micellar Interface : The Preparation of BzPhe-Pro-OMe (47) in Isooctane : Water : AOT : DODCI System :

A solution of AOT (0.44 g, 1 mmol) in Isooctane (10 ml) was admixed with H_2O (0.2 ml) and to the resulting clear solution was added, BzPhe (0.134 g, 0.5 mmol) followed by, under stirring. DODCI (0.273 g, 0.5 mmol). After 0.25 h, this solution was added in one lot to stirred Pro-OMe (0.0645 g, 0.5 mmol) - prepared as described in EXPERIMENT XXIV.f. The reaction mixture was left stirred for 2 d and worked up as described in EXPERIMENT XLI. The residue was subjected to preparative tlc using PhH as developer gave 0.064 g (34%) of BzPhe-Pro-OMe (47), as thick syrup, identical in all respects to an authentic sample.[71]

ir : ν_{max}(neat) cm^{-1} : 3340 (-NH), 1750 (ester), 1640, 1545 (amide).

nmr : $\delta(CDCl_3)$: 2.0 (m, 4H, $(C\underline{H}_2)_2$), 3.23 (d, 2H, -CHC$\underline{H}_2$Ph), 3.66(s, 3H, COOC$\underline{H}_3$), 3.9(m, 2H, -N-C$\underline{H}_2$), 4.4(m, 1H, -CH), 5.2(m, 1H, -CH), 7.0 - 7.9 (m, 11 H, -N$\underline{H}$, 2 x Ph).

XLVI. The Demonstration Of Peptide Bond Formation At the Micellar Interface : The Preparation Of BzGlu(γ -OMe)-Glu(γ -OMe)-OMe (33) in Isooctane : Water : AOT : DODCI System :

A solution of AOT (0.44g, 1 mmol) in Isooctane (10 ml) was admixed with H_2O (0.2 ml) and to the resulting clear solution was added, BzGlu(γ -OMe)-OH (0.132 g, 0.5 mmol) follwed by under stirring, DODCI (0.273 g, 0.5 mmol). After 0.25 h, this solution was added in one lot to stirred Glu-diOMe (0.087 g, 0.5 mmol) - prepared as described in EXPERIMENT XXIV.h. The reaction mixture was left stirred for 2 d and worked up as described in EXPERIMENT XLI. The residue was chromatographed on silica gel. Elution with EtOAc gave 0.028 g

(13%) of (33) as sticky solid, identical in all respects to an authentic sample (EXPERIMENT XXVIII.d).

XLVII. The Demonstration Of Peptide Bond Formation At The Micellar Interface : The Preparation Of BzAsp(β -OMe)-Asp(β -OMe)-OMe (50) in Isooctane : Water : AOT : DODCI System :

A solution of AOT (0.66 g, 1.5 mmol) in Isooctane (15 ml) was admixed with H_2O (0.3 ml) and to the resulting clear solution was added, BzAsp(β -OMe)-OH (0.188 g, 0.75 mmol) followed by, under stirring, DODCI (0.409 g, 0.75 mmol). After 0.25 h, this solution was added in one lot to stirred Asp-diOMe (0.121 g, 0.75 mmol) - prepared as described in EXPERIMENT XXXIV.b. The reaction mixture was left stirred for 2 d and worked up as described in EXPERIMENT XLI. The residue was subjected to preparative tlc using EtOAc as developer to give 0.034 g (11%) of (50), mp. 136°C. (lit.[70] mp.135°), identical in all respects to an authentic sample.

ir : ν_{max}(KBr) cm^{-1} : 3300 (-NH), 1730 (ester), 1645, 1530 (amide).

nmr : δ ($CDCl_3$) : 2.9 (m, 4H, 2 x $CHC\underline{H}_2COOMe$), 3.65, 3.75 (3H, 6H, 2 x $COOC\underline{H}_3$), 4.9 (m, 2H. tert.protons), 7.4-7.9 (m, 7H, aromatic + 2 x NH).

XLVIII. The Demonstration Of Preferential Peptide Bond Formation At The Micellar Interface : The Reaction Of BzGlu(γ -OMe)-OH with Leu-OMe and Glu(γ -OMe)-OMe in Isooctane : H_2O : AOT : DODCI System :

A solution of AOT (0.66 g, 1.5 mmol) in Isooctane (15 ml) was admixed with H_2O (0.3 ml) and to the resulting clear solution was added, BzGlu(γ -OMe)-OH (0.198 g, 0.75 mmol) followed by, under stirring, DODCI (0.409 g, 0.75 mmol).

After 0.25 h, this solution was added in one lot to stirred mixture of LeuOMe (0.119 g, 0.75 mmol) and Glu-diOMe (0.131 g, 0.75 mmol) - prepared, respectively, as described in EXPERIMENTS XXIII.b. and XXIV.h. The reaction mixture was left stirred for 2 d and worked up as described in EXPERIMENT XLI. The residue was chromatographed on silica gel. Elution with PhH : EtOAc (70:30) gave 0.068 g (23%) of BzGlu(γ -OMe)-Leu-OMe (32) as sticky solid and small amounts of BzGlu-(γ -OMe)-Glu-diOMe (33). The spectral data of the products (32) and (33) were identical to those obtained from EXPERIMENTS XXVIII.c. and XXVIII.d.

XLIX. The Demonstration Of Preferential Peptide Bond Formation At The Micellar Interface : The Reaction Of BzAsp(β -OMe)-OH with Leu-OMe and Asp(β -OMe)-OMe in Isooctane : H_2O : AOT : DODCI System :

A solution of AOT (0.44 g, 1 mmol) in Isooctane (10 ml) was admixed with H_2O (0.2 ml) and to the resulting clear solution was added, BzAsp(β -OMe)-OH (0.125 g, 0.5 mmol) followed by, under stirring, DODCI (0.273 g, 0.5 mmol). After 0.25 h, this solution was added in one lot to stirred mixture of LeuOMe (0.073 g, 0.5 mmol) and Asp-diOMe (0.081 g, 0.5 mmol) - prepared, respectively, as described in EXPERIMENTS XXIII.b. and XXXIV.b. The reaction mixture was left stirred for 2 d and worked up as described in EXPERIMENT XLI. The residue was chromatographed on silica gel. Elution with PhH : EtOAc (70:30) and (1:1) gave respectively 0.036 g (19%) of BzAsp(β -OMe)-LeuOMe (45) as thick syrup, and 0.008 g (4%) of BzAsp(β -OMe)-Asp-diOMe (50).

Compounds (45) and (50) were found to be identical in all respects to authentic samples.

(45) : Thick syrup.

ir : ν_{max}(neat) cm^{-1} : 3330, 3300 (-NH), 1750 (ester), 1650, 1555 (amide).

nmr : δ ($CDCl_3$) : 1.0 (d, 6H, $CH(\underline{CH}_3)_2$), 1.25 (m, 1H, $CH_2\underline{CH}(CH_3)_2$), 1.65 (m, 2H, $CH\text{-}\underline{CH}_2CH(CH_3)_2$), 3.0 (m, 2H, $CH\text{-}\underline{CH}_2COOCH_3$), 3.7 (s, s, 6H, 2 x $COO\underline{CH}_3$), 4.6, 4.9 (m, m, 1H, 1H, tert.protons), 7.0-7.9 (m, 7H, aromatic, 2 x NH).

(50) : For spectral data, see EXPERIMENT XLVII.

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44. It may be noted that BzGlu (α -OMe)-OH arises as a minor product (25%) in the oxidation of BzPro-OMe. Attempts to hydrolyze the major product, Bz-pyrogluOMe (61%) to BzGlu(α -OMe)-OH did not succeed. Endeavours to prepare BzGlu(α-OMe)-LeuOMe by treatment of Bz-pyroglu-OMe with LeuOMe did not succeed.

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VITAE

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